ENDOCRINOLOGY

VOLUME 68 APRIL, 1961 NUMBER 4

Copyright © 1961 by the Endocrine Society

THE METABOLIC RATE AND SERUM PROTEIN-BOUND IODINE OF ADRENALECTOMIZED RABBITS MAINTAINED ON REDUCED CORTISONE THERAPY[1]

IRVIN BRODER,[2] BERNARD LANGER,[3] CALVIN EZRIN[4] AND WILLIAM J. HORSEY[5]

Departments of Medicine, Pathology and Surgery, University of Toronto, Toronto, Ontario, Canada

ABSTRACT

The metabolic rate and serum protein-bound iodine were measured in adrenalectomized rabbits maintained on 1.2 mg. of cortisone daily. When this dose was reduced to a level ranging from 0.15 to 0.3 mg. daily, the metabolic rate rose significantly without a corresponding change in serum protein-bound iodine. The possible mechanisms involved are discussed.

EXPERIMENTAL evidence in rabbits suggests that in the presence of a decrease in available adrenal corticoids, there is an increase in thyroid function. Marine and Baumann (1) demonstrated a rise in metabolic rate in the majority of rabbits which survived longer than the initial post-operative period following either bilateral adrenal removal or destruction of the adrenal cortex by freezing. This increase in metabolic rate was not observed after bilateral adrenalectomy in thyroidectomized rabbits (2). More recently, Brown-Grant *et al.* (3) observed a rise in the thyroidal release rate of radioactive iodine when the cortisone maintenance dose of adrenalectomized rabbits was reduced.

The following experiment was designed to further clarify the relationship between thyroid and adrenal function in rabbits. Metabolic rate and serum protein-bound iodine were studied in adrenalectomized rabbits receiving control and reduced doses of cortisone.

Received May 31, 1960.

[1] This work was supported by a grant-in-aid from the National Research Council of Canada.

[2] Helen Vanderveer Fellow, 1957–58.

[3] Research Fellow, National Research Council of Canada, 1958–59.

[4] Associate, Department of Medicine, University of Toronto.

[5] Associate, Department of Surgery, University of Toronto.

METHODS

Mature male rabbits of mixed strain, weighing from 2.0 to 4.0 kg. were used. Bilateral adrenalectomy was performed in 8 animals through a transabdominal approach. Adequacy of operation was checked by gross inspection at autopsy. The lymphopenic response to stress was assessed as well in 4 animals and found to be absent (4, 5). The rabbits were maintained on a daily dose of 1.2 mg. of cortisone acetate,[6] administered subcutaneously.

There was an interval of at least 2 weeks between adrenalectomy and the beginning of any study. Of the 8 animals, 3 served as controls and received 1.2 mg. of cortisone daily for the duration of the experiment. Six studies were carried out with the remaining 5 animals. Each of these experiments was divided into 3 periods, 4 to 6 weeks in length, during which at least 9 metabolic rate and generally 5 serum protein-bound iodine determinations were carried out. During the first and third periods, the animals were maintained on 1.2 mg. of cortisone daily. The dose was reduced to a level which ranged between 0.15 and 0.3 mg. daily during the second period. In each animal, the values for metabolic rate and serum protein-bound iodine obtained during periods one and three were combined for comparison with those of period two.[7]

The metabolic rate (M.R.) was calculated from the oxygen consumption measured in a closed system apparatus over a 40-minute interval. The value for M.R. was expressed in terms of calories per square metre per 24 hours.[8] The rabbits were fasted and kept at a constant environmental temperature (28–30° C) for 24 hours prior to each determination. They were then weighed and had their rectal temperatures measured. On the whole, the rabbits rested quietly while their oxygen consumption was being determined.

The serum protein-bound iodine (PBI) was determined by the method of Barker *et al.* (7). Each sample was measured in either duplicate or triplicate, and expressed in terms of μg per 100 ml.

The significance of differences was assessed with Student's "t" test (8). P was considered to be significant if .01 or less.

RESULTS

There was no appreciable change in rectal temperature or activity during the measurement of M.R. in all of the animals during the experimental interval. In general, the body weights tended to rise gradually by a small increment in both the control and test groups.

The results of M.R. and PBI determinations are summarized in Tables 1 and 2. In the 3 control animals no significant change was seen in the M.R., whereas the PBI remained constant in 1 animal, fell in another, and rose in the third.[9]

In each of the test animals there was a significant rise in M.R. during the period of reduced cortisone dosage, and no corresponding change in

[6] Merck, Sharp and Dohme.

[7] Two separate studies were carried out with rabbit No. 15. During the second of these, it was continued in period two for 10 weeks on a cortisone maintenance dose of 0.2 mg. daily. At the end of this time the animal died, before the maintenance dose could be increased to the control level. There are no data for a third period in this study.

[8] Surface area (cm.^{2}) = 56.33 × weight (gm.)$^{0.436}$ (6).

[9] From the random changes in PBI of the control animals, the degree of possible biological and/or methodological variation of this index in rabbits can be appreciated.

TABLE 1. METABOLIC RATE AND SERUM PROTEIN-BOUND IODINE OF CONTROL ANIMALS

Rabbit	Periods 1 and 3, cortisone 1.2 mg./day		Period 2,* cortisone 1.2 mg./day		M.R.		PBI	
	M.R.†	PBI‡	M.R.	PBI	Change	P	Change	P
13	870 ± 65§	2.0 ± 0.3	907 ± 42	1.8 ± 0.3	+37	.05	−0.2	.3
19	653 ± 69	3.1 ± 0.7	625 ± 57	2.2 ± 0.5	−28	.2	−0.9	.01
23	666 ± 76	2.4 ± 0.4	681 ± 44	3.0 ± 0.4	+15	.6	+0.6	.01

*This period corresponds to that of cortisone deprivation in test animals.
† Cal. per sq. m. per 24 hours.
‡ μg per 100 ml.
§ Mean and standard deviation.

PBI. A representative graph of M.R. results for one of the test animals is shown in Figure 1. It is apparent that the M.R. rose within 1 week of decreasing the dose of cortisone, and returned to the previous rate within 1 week of increasing the dose to its former level.

DISCUSSION

On examining Tables 1 and 2 it becomes apparent that there was a moderate spread in the M.R. and PBI data of individual rabbits during each period of the experiment. It may be asked if the rise observed in the M.R. of the test animals during the period of reduced cortisone therapy might be due to variations in unrecognized and uncontrolled biological factors rather than being related to the known change in therapy. It was established that the rise in M.R. was not only consistent, but also that it was statistically significant. No similar change was found in contemporary control animals which were similarly treated in all respects other than being maintained on a constant dose of cortisone. Rectal temperatures, body weights and activity of the animals showed no constant change which might account for the rise in M.R. of the test group. As well, there was no consistent associated change in the PBI.

It appears most likely that the reduction in steroid therapy was responsible for the rise in M.R. There are two apparent mechanisms by which this might occur without a corresponding change in PBI:

i) increased rate of release of hormone from the thyroid gland, with an

TABLE 2. METABOLIC RATE AND SERUM PROTEIN-BOUND IODINE OF TEST ANIMALS

Rabbit	Periods 1 and 3, cortisone 1.2 mg./day		Period 2, cortisone 0.15–0.3 mg./day		M.R.		PBI	
	M.R.	PBI	M.R.	PBI	Change	P	Change	P
14	773 ± 82	1.7 ± 0.3	921 ± 85	1.8 ± 0.3	+148	.001	+0.1	.6
15	707 ± 60	1.8 ± 0.3	794 ± 90	1.4 ± 0.3	+ 87	.01	−0.4	.05
15*	777 ± 64	2.2 ± 0.3	889 ± 78	2.2 ± 0.2	+112	.001	0	—
24	726 ± 59	2.5 ± 0.3	818 ± 75	2.3 ± 0.1	+ 92	.01	−0.2	.2
25	834 ± 90	2.9 ± 0.4	979 ± 84	3.2 ± 0.1	+145	.001	+0.3	.05
28	718 ± 75	2.9 ± 0.5	839 ± 89	3.2 ± 0.4	+121	.001	+0.3	.2

* This animal was studied on two occasions.

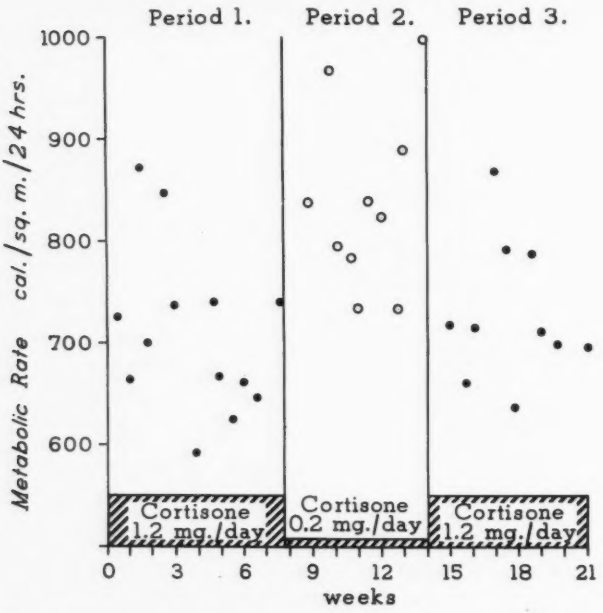

FIG. 1. Metabolic rate of rabbit No. 28 while receiving control and reduced cortisone maintenance therapy.

equivalent increase in the rate at which the hormone leaves the serum being accompanied by a rise in its metabolic activity;

ii) enhanced biological effectiveness of a fixed amount of thyroid hormone.

Several observations support the first hypothesis. Marine and Baumann (1) demonstrated a rise in metabolic rate following either adrenal destruction or removal. This did not occur in animals which were previously thyroidectomized (2). These findings suggest that the rise in metabolic rate was related to a functioning thyroid gland. Brown-Grant *et al.* (3) observed an increased thyroidal release rate of radioactive iodine in adrenalectomized rabbits maintained on a reduced dose of cortisone over an interval of several days. It is assumed that this reflects an increased rate of hormone output from the thyroid gland and that this would persist for 4 to 6 weeks if it were possible to follow this index over longer intervals. The present observations indicate that when reduced cortisone replacement therapy is administered over an interval of several weeks, the PBI remains constant although the M.R. becomes elevated. These findings suggest that if there is an increased output of thyroid hormone while the animals are receiving reduced cortisone therapy, there may be an equivalent increase in the rate at which the hormone leaves the serum, and an associated rise in its metabolic activity.

There is less direct evidence in favor of thyroid hormone having an enhanced biological effectiveness in the presence of reduced cortisone maintenance therapy. It has been shown in animals given large doses of thyroid hormone or thyrotropin, that the negative nitrogen balance (9) and weight loss (10, 11) both become less marked if adrenal extract or cortisone is administered as well. Bastenie and Kowalewski (12) demonstrated that whereas thyroxin administration in hypophysectomized rats produces a rise in oxygen consumption, those treated with ACTH as well show no such change. These observations suggest that glucocorticoids may in some manner interfere with the peripheral effectiveness of thyroid hormone. It is possible that in the presence of a reduced amount of glucocorticoid the biological effectiveness of a fixed amount of thyroid hormone may become enhanced.

Although the present observations do not permit a definite conclusion to be drawn, the evidence favors the first hypothesis. It is possible that both or other mechanisms may contribute to the observed changes. Studies are in progress to clarify this further.

Acknowledgments

The authors are indebted to Norma Huber, Ignatia Leewens and Denise De Pinto for technical assistance.

REFERENCES

1. Marine, D. and E. J. Baumann: *Am. J. Physiol.* **57:** 135. 1921.
2. Marine, D. and E. J. Baumann: *Am. J. Physiol.* **59:** 353. 1922.
3. Brown-Grant, K., G. W. Harris and S. Reichlin: *J. Physiol.* **126:** 41. 1954.
4. Dougherty, T. F. and A. White: *Endocrinology* **35:** 1. 1944.
5. Colfer, H. F., J. de Groot and G. W. Harris: *J. Physiol.* **111:** 328. 1950.
6. Smuts, D. B.: *J. Nutr.* **9:** 403. 1935.
7. Barker, S. B., M. J. Humphrey and M. H. Soley: *J.C.I.* **30:** 55. 1951.
8. Bernstein, L. and M. Weatherall: Statistics for Medical and Other Biological Students. Livingstone: Edinburgh, 1952.
9. Koelsche, G. A.: *Proc. St. M. Mayo Clinic* **9:** 55. 1934.
10. Kinsell, L. W., F. O. Zillesson A. M. Smith, and J. Palmer: *Endocrinology* **30:** 221. 1942.
11. Aterman, K.: *Acta Endocrinol.* Suppl. 20. 1954.
12. Bastenie, P. A. and K. Kowalewski: *Compte. Rend. Soc. de Biol.* **145:** 772. 1951.

THYROXINE ANALOGUES AND CHOLESTEROL METABOLISM: THE PREVENTION AND REVERSAL OF ELEVATED LIVER CHOLESTEROL AND LIPID CONCENTRATIONS IN THE CHOLESTEROL FED RAT BY DIETARY3,5 DIIODOTHYROACETIC ACID[1]

W. R. RUEGAMER AND F. R. SILVERMAN[2]

Radioisotope Laboratories, Veterans Administration Hospital and the Department of Biochemistry, State University of New York College of Medicine, Syracuse, New York

ABSTRACT

It was found that a dietary concentration of 5 mg.% Diac (3,5 diiodothyroacetic acid) is optimal for the prevention of elevated liver cholesterol and lipid concentrations in the rat fed a high fat diet containing 1% cholesterol. Diac also reduced previously elevated liver cholesterol and lipid concentrations to chow diet control values within three weeks. Growth rates were not affected at the 5 mg.% level, but a progressive decrease in growth and increase in oxygen consumption rates occurred at dietary levels ranging from 25 to 100 mg.%. Feeding the high fat diet containing 1% cholesterol and 5 mg.% Diac for five months produced a vitamin K deficiency. This condition could be corrected by dietary supplementation with vitamin K and no further injurious effects were observed.

A SURVEY has been made of a number of thyroxine analogues for a compound which will produce a maximum reduction in tissue cholesterol and lipid concentrations without significantly affecting other biological parameters such as oxygen consumption and growth (1, 2). Of the compounds tested, 3,5 diiodothyroacetic acid (Diac)[3] appeared to be the most preferential in this respect, and deserves further consideration as a potential agent for the control of cholesterol metabolism in persons predisposed towards atherosclerosis (3). A more extensive study of this compound has been made in cholesterol-fed rats, and the results of these investigations constitute the subject matter of this report.

Received June 22, 1960.

[1] This work was presented in part at the IVth International Goiter Conference in London, England.

[2] The authors are grateful to the Warner Lambert Research Institute for a grant which partially defrayed the expenses incurred while carrying out this project.

[3] Supplied as compound number W 1502 by Warner Lambert Research Institute, Morris Plains, New Jersey.

METHODS

Weanling male Albino rats (Holtzman) were housed by pairs in wire mesh-bottomed cages in an air conditioned animal room maintained at approximately 25° C. Except for the first experiment in which the cholesterol and cholic acid content of the diet was varied, the animals were fed a basic 19% casein, 29% lard, 1% cholesterol diet (Table 1). Food and water were supplied *ad libitum*. The animals were weighed at weekly intervals for a period of three weeks, at the end of which time measurements were made of the individual oxygen consumption rates where such data appeared pertinent. After

TABLE 1. PERCENTAGE COMPOSITION OF THE PURIFIED HIGH FAT-CHOLESTEROL DIET (HFD-C)

	Gm. %
Casein	19.00
Glucose	47.40
Lard	29.00
Salts[1]	3.00
Cholesterol	1.00
Cod liver oil[2]	0.30
Alpha tocopherols	0.03
Water soluble vitamins	**Mg. %**
Thiamin	0.375
Riboflavin	0.470
Pyridoxine	0.375
Nicotinic acid	1.875
Pantothenic acid	1.875
Choline chloride	187.500
Folic acid	0.375
Vitamin B_{12}	0.015
Biotin	0.094
Inositol	93.750

[1] Phillips & Hart Salt Mixture.
[2] 850 I.U. Vitamin A per gram and 85 I.U. Vitamin D per gram.

the completion of these determinations, the animals were anesthetized with ether and heparinized blood samples were collected by heart puncture for plasma cholesterol measurements. Liver specimens were also obtained for liver cholesterol and total lipid determinations. These values were calculated as per cent of fresh weight. The methods and procedures employed have been described in a previous communication (2).

RESULTS

Selection of diet

In our first experiments (1, 2) a purified diet low in protein (16% casein), high in saturated fat (29% lard), and containing 1% cholesterol plus 0.5% cholic acid was employed. It is known that these factors enhance hypercholesterolemia in the rat (4–10), and feeding this diet elevated the plasma and liver cholesterol concentrations from average chow diet control values of 77 and 300 mg.% to 438 and 6,350 mg.%, respectively. However, because of the introduction of so many dietary variables, interpretation of the effects of Diac upon tissue cholesterol and lipid concentrations is difficult. Consequently, the protein content of the basic diet was raised to 19%, and the effects of various dietary levels of cholesterol and cholic acid were evaluated with the intention of selecting a more physiological

diet. The regima shown in Table 2 were fed to eight groups of eight weanling male rats for three weeks, after which the animals were sacrificed and plasma and liver cholesterol and liver lipid concentrations were determined. Cholesterol and cholic acid were incorporated into the basic diet at the expense of the carbohydrate. It can be seen from Table 2 that the basic high fat diet (HFD) without additives produced very little effect on liver cholesterol and lipid values. However, plasma cholesterol concentrations were elevated. The addition of 0.1%, 0.5%, and 1.0% cholesterol to the diet produced a progressive increase in liver cholesterol, and the 0.5% and

TABLE 2. EFFECT OF FEEDING VARIOUS LEVELS OF CHOLESTEROL AND CHOLIC ACID FOR 3 WEEKS ON AVERAGE WEIGHT GAINS AND TISSUE CHOLESTEROL AND LIPID CONCENTRATIONS

Regimen	3-Week wt. gain (gm.)	Plasma cholesterol (mg.%)	Liver cholesterol (mg.%)	Liver lipid (gm.%)
Purina chow	122 ± 5.8†	70 ± 4	425 ± 17	11.3 ± 1.32
HFD*	113 ± 6.8	122 ± 12	437 ± 20	12.4 ± 1.36
HFD + 0.1% cholesterol	103 ± 7.1	93 ± 4	902 ± 42	12.0 ± 2.14
HFD + 0.5% cholesterol	103 ± 4.0	108 ± 13	2050 ± 80	18.3 ± 2.45
HFD + 1.0% cholesterol	109 ± 6.1	114 ± 9	2760 ± 110	17.5 ± 2.20
HFD + 0.1% cholesterol + 0.5% cholic acid	101 ± 8.1	122 ± 12	2840 ± 115	19.5 ± 1.39
HFD + 0.5% cholesterol + 0.5% cholic acid	91 ± 6.4	161 ± 12	4760 ± 103	22.3 ± 1.9
HFD + 1.0% cholesterol + 0.5% cholic acid	115 ± 8.2	217 ± 16	6740 ± 250	29.7 ± 2.0

* The basic high fat diet (HFD) had the same composition as that of the diet shown in Table 1 except that cholesterol was omitted.

† The values given are arithmetic means together with standard deviations.

1% levels also raised liver lipid values. The addition of cholic acid to these regima produced a still greater elevation in tissue cholesterol and lipid concentrations, and the extent of the effect appears to be a function of the concentration of cholesterol present in the diet. Since a marked elevation in liver cholesterol and lipid concentrations could be produced with the 1% cholesterol diet without cholic acid, it was decided to employ this regimen (Table 1) in succeeding experiments.

Relative effects of Diac when injected or fed

In the initial screening experiments, all of the thyroxine derivatives tested were injected intraperitoneally at a concentration of 0.5 micromoles per 100 gm. body weight per day (1, 2). An experiment was performed next to determine the relative activity of oral Diac on the prevention of elevated liver cholesterol and lipid concentrations. Four groups of 6 rats each were placed on the regima shown in Table 3. The basic high fat-1% cholesterol diet (HFD-C) shown in Table 1 was employed. The Diac-injected group received daily intraperitoneal injections of 500 micrograms

of Diac per 100 gm. body weight per day, and the Diac and thyroxine (T_4) fed groups received these compounds at a level of 5 mg.% in the diet. The Diac-fed animals averaged 440, 470 and 460 μg Diac per 100 gm. body weight per day over the first, second and third weeks of the experiment respectively. Therefore, the Diac fed animals received approximately the same amount of Diac as the injected group, although it must be remembered that the injected Diac was given as a single daily administration. Thus, if Diac turnover is rapid, exposure to Diac was effected for only a small portion of each 24-hour period, whereas the fed material was in essence administered over a much longer interval. Tested under these conditions, orally administered Diac was more effective than injected Diac in the prevention of elevated liver cholesterol and lipid concentrations. Stasilli and Kroc (11) also found that Diac is more effective in both

TABLE 3. THE RELATIVE EFFECTS OF ORAL AND INJECTED DIAC

Regimen*	Avg. 3-week weight gain (gm.)	Avg. plasma cholesterol (mg.%)	Avg. liver cholesterol (mg.%)	Avg. liver lipid (gm.%)
Purina chow	118 ± 10†	63 ± 8	286 ± 57	10.4 ± 1.3
HFD-C	103 ± 12	122 ± 13	3940 ± 113	19.0 ± 2.1
HFD-C + Diac injected	101 ± 9	113 ± 10	1970 ± 265	13.1 ± 2.0
HFD-C + Diac fed	117 ± 9	108 ± 16	385 ± 84	8.3 ± 1.2

* Diac was injected intraperitoneally at a concentration of 1.0 micromole per 100 grams body weight per day. In the feeding experiment, Diac was incorporated into the diet at a concentration of 5 mg.%.

† The values given are arithmetic means together with standard deviations.

calorigenic and goiter-prevention tests when administered orally in the diet or by stomach tube than when given subcutaneously. Therefore, it was decided to feed rather than inject Diac and other thyroxine-like derivatives in subsequent studies.

Optimal concentration of Diac required for the prevention of high liver cholesterol and lipid concentrations

In the next two experiments, various concentrations of Diac were fed to several groups of 6 weanling male rats (Table 4). Oxygen consumption rates were determined in animals receiving the higher levels of Diac, and all animals were sacrificed for tissue analysis at the end of three weeks. Individual food consumption rates were not measured, but it was observed that all groups of animals on the semipurified diets had disposed of approximately the same amount of food during the experimental period. Therefore, the data presented in Table 4 indicate that the 5 mg.% level of Diac effectively prevented the elevation of liver cholesterol and lipid concentrations. Moreover, Diac levels above 10 mg.% produced no greater effect on cholesterol concentrations, and they impaired growth and oxygen consumption rates. The effectiveness of Diac was sharply reduced at levels

TABLE 4. DETERMINATION OF THE OPTIMAL CONCENTRATION OF DIAC REQUIRED IN THE DIET FOR THE PREVENTION OF ELEVATED LIVER CHOLESTEROL AND LIPID CONCENTRATIONS

Regimen	Average weight gain per 3 weeks (gm.)	Average liters $O_2/M^2/Hr.$	Average plasma cholesterol (mg.%)	Average liver cholesterol (mg.%)	Average liver lipid (gm.%)
Purina chow	118±11*	—	74± 5	384± 28	11.3±1.2
HFD-C	108± 8	—	108±10	2770±116	16.2±1.0
HFD-C+0.4 mg.% Diac	115± 9	—	104±10	2400±298	15.8±0.9
HFD-C+1.0 mg.% Diac	122±15	—	107± 7	2110±239	15.6±1.4
HFD-C+2.5 mg.% Diac	102±10	—	98±10	1430±130	—
HFD-C+5.0 mg.% Diac	102±10	—	106± 8	622± 48	9.8±0.8
Purina chow	126±11	6.2±0.6	82± 8	244± 18	10.6±1.1
HFD-C	117±16	6.5±1.0	136±11	2241±185	15.3±1.5
HFD-C+ 5 mg.% Diac	103±11	7.4±0.9	124±12	501± 42	9.4±1.2
HFD-C+ 10 mg.% Diac	93± 9	7.4±1.1	154±13	535± 45	9.6±0.8
HFD-C+ 25 mg.% Diac	91±10	8.8±1.2	169±10	569± 43	9.9±0.9
HFD-C+ 50 mg.% Diac	73±10	9.5±1.1	150±13	568± 42	8.9±1.1
HFD-C+100 mg.% Diac	57± 6	9.9±1.1	153±11	359± 40	9.6±1.0

* The values given are arithmetic means together with standard deviations.

below 5 mg.%. Therefore, a Diac concentration of 5 mg.% in the diet was considered optimal.

Diac reversal of high liver concentrations of cholesterol and lipid

Except for our initial experiments (1, 2) which were conducted under different experimental conditions, all of the experiments described have been concerned with the prevention of elevated liver cholesterol and lipid concentrations in cholesterol fed animals. In the next two experiments (Table 5), the reversal of previously elevated cholesterol and lipid concentrations was attempted through Diac administration. In the first experiment, 24 weanling male rats were started on the high fat diet plus 1% cholesterol (HFD-C). At the end of three weeks, 8 animals were sacrificed and the same biological parameters were measured as in previous experiments. With the successful induction of high liver cholesterol and lipid concentrations in these animals (Table 5, Group 1), the remaining 16

TABLE 5. REVERSAL OF ELEVATED LIVER CHOLESTEROL AND LIPID CONCENTRATIONS

Group	Number of weeks on			Avg. plasma cholesterol (mg.%)	Avg. liver cholesterol (mg.%)	Avg. liver lipid (gm.%)
	HFD-C	HFD-C-D				
Expt. 1						
1	3	0		90± 5*	2770±275	18.3±1.0
2	6	0		93± 5	3290±152	19.3±1.1
3	3	3		75± 4	1750± 83	11.1±1.1
Expt. 2	HFD-C-CA	HFD	HFD-D			
4	3	0	0	222±18	4728±372	19.8±2.0
5	3	1	0	163±15	5156±419	18.3±1.3
6	3	3	0	123±11	2147±204	16.2±1.0
7	3	0	1	147±12	2402±200	11.3±0.9
8	3	0	3	140± 8	325± 67	10.9±1.1

* The values given are arithmetic means together with standard deviations.

animals were subdivided into two equal groups. Group 2 was continued on the diet without Diac (HFD-C) and Group 3 received a supplement of 5 mg.% Diac in the diet (HFD-C-D). At the end of another three weeks, all animals were sacrificed, and the same tissue analyses were performed. The results, summarized in Table 5, indicate that a marked reduction in tissue cholesterol and lipid concentrations was effected by Diac administration even in the presence of continued cholesterol feeding. Liver cholesterol values were not reduced to those observed in animals receiving a chow diet (Tables 2–4), but plasma cholesterol and liver lipid values were reduced to essentially the control diet range.

In the second experiment, 40 weanling male rats were fed the high fat diet containing 1% cholesterol and 0.5% cholic acid (HFD-C-DA) for a period of three weeks. This regimen produced a marked increase in the average tissue cholesterol and lipid concentrations of 8 animals sacrificed for tissue analysis (Table 5, Group 4). The remaining animals were subdivided into four groups of eight rats each. Groups 5 and 6 were placed back on the high fat diet without added cholesterol (HFD), and Groups 7 and 8 received the same regimen plus 5 mg.% Diac (HFD-D). After one week, the liver cholesterol concentration of the Diac supplemented group (Group 7) had dropped by one-half and the liver lipid concentration was reduced to a level frequently observed in control animals fed chow diet (Tables 2–4). The unsupplemented group (Group 5) still had liver cholesterol and lipid values comparable to those seen in Group 4. After three weeks on these regima, the average liver cholesterol concentration of the Diac supplemented group (Group 8) had returned to chow diet control levels, whereas the unsupplemented group (Group 6) still had very high cholesterol and lipid values. No demonstrable difference in the plasma cholesterol was evident between the Diac supplemented and unsupplemented groups after three weeks.

Effect of long-term feeding of Diac to rats

It was observed that the feeding of Diac at levels of 25–100 mg.% in the diet produced a decreased growth rate and an elevated oxygen consumption rate in animals receiving these regima (Table 4). Therefore, it became important to determine the relative degree of Diac toxicity when fed at the optimal concentration of 5 mg.% for an extended period of time. Accordingly, three groups of twenty rats each were started on the following: 1) Purina chow diet, 2) the standard high fat diet plus 1% cholesterol (HFD-C) and 3) the same purified diet plus 5 mg.% Diac. At the end of approximately 7 weeks, two rats receiving the Diac supplemented ration died. During the course of the next three weeks, a total of five more rats succumbed on this diet and two animals in the HFD-C group also died. All animals apparently died as a result of severe hemorrhage in the lower peritoneal cavity. In formulating the synthetic diet used in these studies,

vitamin K had been omitted since it was believed that the intestinal flora of the rat synthesizes and makes available sufficient amounts of Vitamin K to satisfy the rat's own requirements.

However, in view of these observations, three rats from each of the experimental groups were sacrificed, and blood specimens were obtained and assayed for prothrombin content according to the procedure of Quick (12). Prothrombin times averaged 15.0, 17.3 and 24.5 seconds for the chow, HFD-C and HFD-C+5 mg.% Diac groups, respectively. Vitamin K in the form of menadione sodium bisulfite was then included in both of the purified diets at a level of 0.5 mg.%. After one week on this supplement, prothrombin times for all groups averaged approximately 15 seconds. Therefore, it might be concluded that a vitamin K deficiency was probably responsible for the hemorrhage and death occurring in animals receiving the purified diets.

Five animals in each group were continued on their respective regima except for the addition of 0.5 mg.% of menadione sodium bisulfite to the two groups receiving the purified diets. At the end of five months, these animals were sacrificed and the usual parameters were measured. From the data summarized in Table 6, it can be seen that those animals receiv-

TABLE 6. EFFECT OF FEEDING 5 MG.% DIAC IN THE HIGH FAT-CHOLESTEROL DIET FOR A PERIOD OF 5 MONTHS

Regimen	Average weight gain (gm.)	Average plasma cholesterol (mg.%)	Average liver cholesterol (mg.%)	Average liver lipid (gm.%)	Average aorta cholesterol (mg.%)
Purina chow	400 ± 38*	94 ± 8	201 ± 21	9.5 ± 0.3	112 ± 19
HFD-C	460 ± 51	103 ± 8	7033 ± 255	28.7 ± 1.8	109 ± 16
HFD-C + 5 mg.% Diac	424 ± 41	87 ± 7	1184 ± 114	13.0 ± 0.7	116 ± 7

* The values given are arithmetic means together with standard deviations.

ing 5 mg.% Diac for five months showed significantly lower plasma and liver cholesterol and liver lipid concentrations ($P = 0.01$ for plasma differences and $P < 0.001$ for liver cholesterol and lipid differences). Diac effected more than a six-fold reduction in liver cholesterol values, and maintained the plasma cholesterol and liver lipid values at levels fairly comparable to those seen in chow diet controls (Table 6). Aortic cholesterol concentrations were the same for all groups, and the feeding of cholesterol over this period of time apparently did not result in the deposition of cholesterol in the aorta.

DISCUSSION

It is known (13) that the amount of cholesterol synthesized in the body is inversely related to the amount ingested in the diet, and that the feeding of 1% cholesterol virtually halts the endogenous synthesis of cholesterol. Therefore the effects produced by Diac in the present set of experiments

are largely concerned with alterations in exogenous cholesterol metabolism. Nevertheless, it is apparent from the data that an optimal concentration of dietary Diac will prevent the development of abnormally high cholesterol and lipid concentrations in the cholesterol fed rat. Diac fed animals generally do not have values equal to those seen in chow diet controls, but they are maintained at a very much lower level than those of the cholesterol-fed animals (Tables 3, 4 and 6). On the other hand, plasma cholesterol levels were not markedly reduced by Diac feeding. The explanation for this may be that Diac does not suppress the amount of dietary cholesterol absorbed (14), and consequently high plasma cholesterol concentrations merely reflect the absorption of dietary cholesterol from the gastrointestinal tract and transport to storage sites such as the liver.

Diac appears to be very effective in mobilizing liver deposits of cholesterol and lipid, although final assessment of this conjecture must await isotopic studies. In the first mobilization experiment (Table 5), marked reductions in both cholesterol and lipid concentrations were brought about by Diac administration, even in the presence of continued cholesterol feeding (Group 3). In the second experiment, cholesterol feeding was discontinued (Table 5), and Diac reduced the liver concentrations of cholesterol and lipid to chow diet control values within three weeks (Group 8). Control liver cholesterol and lipid concentrations observed in animals receiving the unsupplemented high fat diet (HFD) remained elevated (Group 6). However, the changes in liver cholesterol and lipid concentrations do not describe the over-all effects of Diac on the liver. Over a three-week period, the livers of the HFD control group (Group 6) increased in size by an average of 2.72 gm. whereas the liver weights of Diac-fed animals (Group 8) remained the same. Both groups of animals gained the same amount of body weight during the three-week period. Therefore, when the data are recalculated on the basis of changes in total liver cholesterol and lipid, the control HFD group showed no change whereas the Diac fed animals lost 360 mg. cholesterol and 670 mg. lipid. It was observed in another experiment (14) that approximately 10% of the total liver cholesterol was free and the remainder was bound to fatty acids. If most of the bound cholesterol was esterified to a mixture of C-18 acids (15), a factor of 1.7 might be used to convert bound cholesterol values to cholesterol-fatty acid values. If the fatty acid bound to cholesterol also leaves the liver as a result of Diac administration, then the net decrease in total liver lipid (670 mg.) can be accounted for essentially by the decrease in free cholesterol (36 mg.) plus cholesterol-fatty acid ester (324 mg. $\times 1.7 = 551$ mg.).

As stated above, 2.72 gm. of additional non-lipid substance was laid down in the livers of the HFD control animals (Group 6) during the three-week period. Since 0.67 gm. of lipid left the livers of the Diac-fed animals

(Group 8) without a change in liver weight, this lipid also must have been replaced by 0.67 gm. of another substance. The nature of both of these substances is unknown at present, but it would appear that Diac acts to prevent an increase in liver size as well as to mobilize deposits of liver lipid.

The concept of keeping the tissue stores of cholesterol and possibly other lipids reduced or mobilized through use of a compound such as Diac might represent a sound physiological approach towards the control of atherosclerosis. One objection to the use of substances designed to block endogenous cholesterol synthesis is that such compounds might block other vital metabolic processes or result in the accumulation of intermediary substances having highly undesirable properties. On the other hand, a suitable inhibitor of cholesterol synthesis might be employed in combination with a cholesterol mobilizer such as Diac. Of course this conjecture presupposes that Diac will mobilize aortic cholesterol and lipid deposits as well as it does those of the liver. This possibility could not be tested in the present set of experiments because the aortic cholesterol levels were not elevated under the experimental conditions employed (Table 6).

It was encouraging to find that no injurious effects other than the precipitation of a vitamin K deficiency could be detected in animals fed 5 mg.% Diac over a 5-month period. The acute hemorrhage and resulting death observed in animals receiving the high fat diet containing cholesterol and Diac were overcome by menadione bisulfite administration. Mackenzie, *et al.* (16) have recently described a vitamin K deficiency in rats fed a semipurified diet containing lard as a source of fat. Prothrombin times were markedly elevated in these animals, and approximately one-third of the animals died of severe hemorrhage within ten weeks. Both the elevation of prothrombin times and the hemorrhage could be prevented by the addition of vitamin K to the diet. Barnes, *et al.* (17) and Mameesh, *et al.* (18, 19) have also described the production of a vitamin K deficiency in rats fed a semi-purified diet. Both groups of investigators concluded that coprophagy appears to be the means by which vitamin K synthesized by the intestinal microflora is made available to the rat. When a high fat diet was fed, the feces were odoriferous and the degree of coprophagy was greatly reduced. Consequently, a vitamin K deficiency resulted. The precipitation of a vitamin K deficiency in the present set of experiments can probably be explained in much the same way, although Diac may also have contributed to the effect. Possibly Diac might have accentuated the vitamin K deficiency by increasing the rate of vitamin turnover or absolute requirements for vitamin K.

REFERENCES

1. Ruegamer, W. R., M. Alpert and F. R. Silverman: *Fed. Proc.* **18**: 313. 1959.
2. Ruegamer, W. R., M. Alpert and F. R. Silverman: *Endocrinology* **66**: 160. 1960.
3. Ruegamer, W. R. and F. R. Silverman: *Fed. Proc.* **19**: 16. 1960.

4. Moyer, A. W., D. Kritchevsky, J. B. Logan and H. R. Cox: *Proc. Soc. Exper. Biol. & Med.* **92:** 736. 1958.
5. Pihl, A.: *Acta Physiol. Scand.* **34:** 206. 1955.
6. Beher, W. T. and G. D. Baker: *Proc. Soc. Exper. Biol. & Med.* **98:** 892. 1958.
7. Shapiro, S. L. and L. Freedman: *Am. J. Physiol.* **181:** 441. 1955.
8. Hegsted, D. M., S. B. Andrus, A. Gotsis and O. W. Portman: *J. Nutr.* **63:** 273. 1957.
9. Friedman, M. and S. O. Byers: *Proc. Soc. Exper. Biol. Med.* **78:** 528. 1951.
10. Nath, N., R. Wiener, A. E. Harper and C. A. Elvehjem: *J. Nutr.* **67:** 289. 1959.
11. Stasilli, N. B. and R. L. Kroc. Personal Communication
12. Quick, A. J.: *Am. J. Clin. Path.* **10:** 222. 1940.
13. Tomkins, G. M., H. Sheppard and I. L. Chaikoff: *J. Biol. Chem.* **201:** 137. 1953.
14. Ruegamer, W. R. and F. R. Silverman (unpublished data).
15. Portman, Oscar W. and Frederick J. Stare: *Physiol. Rev.* **39:** 421. 1959.
16. Mackenzie, J. B., R. C. Strader and C. G. Mackenzie: *Fed. Proc.* **18:** 535. 1959.
17. Barnes, R. H. and G. Fiala: *Fed. Proc.* **17:** 470. 1959.
18. Mameesh, M. S. and B. Connor Johnson: *Proc. Soc. Exper. Biol. & Med.* **101:** 467. 1959.
19. Mameesh, M. S. and B. Connor Johnson: *Proc. Soc. Exper. Biol. & Med.* **103:** 378. 1960.

CEREBRAL CORTICAL INHIBITION OF PITUITARY-ADRENAL SECRETION[1]

RICHARD H. EGDAHL[2]

Department of Surgery, Medical College of Virginia, Richmond, Virginia

ABSTRACT

Dogs with chronic adrenal vein cannulas were bilaterally decorticated and 17-hydroxycorticosteroid outputs were determined in the post-operative period. "Resting" corticosteroid outputs in decorticate dogs were markedly elevated compared to normal, with return to baseline levels following Nembutal anesthesia. Sciatic nerve stimulation in the anesthetized decorticated animal resulted in a significant adrenal cortical response which was not further increased by exogenous ACTH. These studies indicate that the cerebral cortex exerts a tonic inhibition on lower central nervous system areas which are involved in the control of ACTH release.

DOGS with removal of all brain tissue down to the pons, except for the isolated pituitary, characteristically demonstrate elevated adrenal venous outputs of 17-hydroxycorticosteroids compared to normal animals (1). Steroid outputs remain elevated during the 2- to 4-day survival period of the animals with brain removal. These findings could be due to the removal of tonic inhibition areas which control ACTH release in the "basal" or "resting" intact animal.

METHOD

Adult male mongrel dogs ranging in weight from 10 to 15 kg. were used in these experiments. Adrenal vein cannulations were carried out according to the technique of Hume and Nelson (2). 17-Hydroxycorticosteroids were measured by the method of Nelson and Samuels (3). Catechol amines were determined by the method of Weil-Malherbe and Bone, as modified by Aranow (4).

Bilateral decortications in dogs bearing adrenal vein cannulas were carried out by performing a craniotomy and removing the cerebral cortices bilaterally with standard neurosurgical techniques. All cortical tissue was removed down to the hippocampus, with special attention directed towards ablation of fragments of the temporal lobes. Hemostasis was obtained with silver clips, and small gelfoam pledgets were used to control residual oozing areas. In the early stages of experiments with decorticate animals, the procedure was performed in two stages. However, it has recently been found that single stage bilateral decortication can be performed with a low mortality and good long range survival, and this is the preferred method at present.

Sterile techniques were used in all operations. Electrodes with insulated lead-off wires were implanted in the sciatic nerve for study of electrical stimulation in these

Received June 27, 1960.

[1] Supported in part by Research Grants from the U. S. Public Health Service (A-2267), (B-2688) and The Commonwealth Fund.

[2] Markle Scholar in Medical Sciences.

animals. A standard protocol consisted of adrenal vein cannulation on the 1st day. Craniotomy without removal of brain tissue was also carried out at the time of adrenal vein cannulation in 3 animals. The next morning representative "resting" adrenal venous samples were obtained and the dog was then anesthetized and decorticated. The day following decortication, control blood samples were taken, following which the animal was again anesthetized with Nembutal. After another series of blood samples had been obtained, electrical stimulation of the sciatic nerve was carried out, followed by administration of 25 units of ACTH intravenously. In two experiments adrenal vein cannulation was carried out one week following bilateral decortication, with blood sample collections and nerve stimulation on the day following cannulation.

RESULTS

A statistical summary of the results in these experiments is given in Table 1. The values for ACTH response were obtained by averaging two adrenal venous corticosteroid output determinations, one made at 5 and one at 10 minutes following exogenous ACTH administration. "Resting"

TABLE 1. STATISTICAL SUMMARY OF EXPERIMENTS IN DOGS WITH ADRENAL VEIN CANNULAS AND BILATERAL DECORTICATION. DECORTICATION WAS CARRIED OUT FOLLOWING ACTH ADMINISTRATION ON THE DAY AFTER CANNULATION (COLUMN #3)

	Day of adrenal vein cannulation	Day after cannulation		Day after decortication			
	1	2	3	4	5	6	7
	ACTH response	Unanesthetized "resting"	ACTH response	Unanesthetized "resting"	Nembutal anesthesia	Nerve stimulation (anesthesia)	ACTH response
Mean	13.6*	1.0	17.7	10.2	2.1	14.1	17.8
Standard Error of Mean	1.7	0.2	2.3	0.8	0.8	2.1	2.2
Number of Experiments	12	14	11	11	11	4	14

* Micrograms/minute, adrenal venous 17-hydroxycorticosteroids.

levels represent averages of five samples obtained at 5-minute intervals in the control period. Values following Nembutal anesthesia were derived by taking averages of five samples obtained at 5-minute intervals following the intravenous administration of 30 mg./kg. of Nembutal. Three output determinations made at 5, 10, and 15 minutes after the start of a continuous nerve stimulation were averaged to give a figure for the post-stimulation adrenal venous corticosteroid response.

There was no significant corticosteroid output difference ($P < 0.2, > 0.1$) between unanesthetized normal "resting" animals and anesthetized "resting" decorticate ones, although both these groups revealed significantly lower outputs than all other groups ($P < .001$). Outputs of the unanesthetized "resting" decorticate dogs were significantly lower than those after ACTH on the days following cannulation or decortication ($P < .01, > .001$). However, unanesthetized "resting" decorticate outputs were not different from those following ACTH on the day of cannulation or following nerve stimulation ($P < 0.2, > 0.1$). This indicates that the "resting" or unstimu-

lated secretion of the decorticate dog is not significantly different from the output following nerve stimulation or ACTH administration on the day of cannulation. Following Nembutal anesthesia, however, the output of corticosteroids falls markedly and is not significantly different from basal outputs the day following adrenal vein cannulation in animals without lesions in the central nervous system. The addition of craniotomy to the procedure of adrenal vein cannulation did not result in elevated corticosteroid outputs on the day following cannulation.

A representative experiment is illustrated in Figure 1. It can be seen that exogenous ACTH administration on the day of adrenal vein cannula-

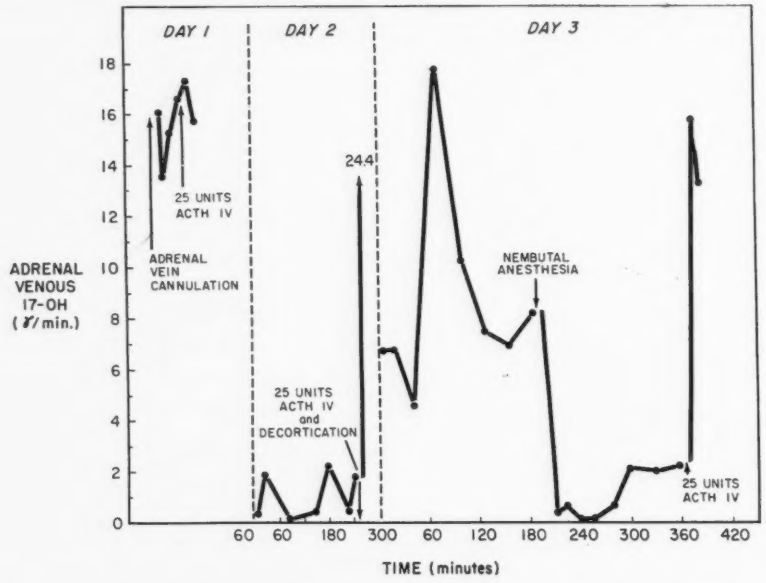

FIG. 1. Adrenal cortical function in decorticate dog (B-143). Note elevated 17-OHCS output, with immediate fall to basal levels following Nembutal administration.

tion does not further increase corticosteroid output from that observed following the operation. The day following adrenal vein cannulation basal corticosteroid outputs are observed. A rise to high levels follows exogenous ACTH administration. On the day following decortication variable but consistently elevated outputs of 17-hydroxycorticosteroids are observed, with an abrupt decline to basal levels following Nembutal anesthesia. The response to exogenous ACTH remained unimpaired.

In Figure 2 are plotted the data from a decortication experiment in which nerve stimulation was carried out. Basal corticosteroid ouputs are recorded the day following adrenal vein cannulation. The day after decortication variably high corticosteroid outputs which fell to low values following Nembutal anesthesia were observed. Nerve stimulation results

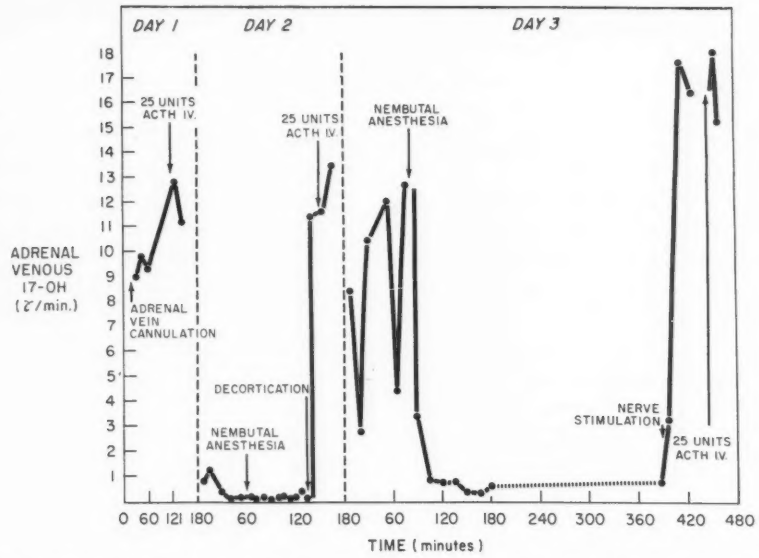

FIG. 2. Same as Figure 1, with addition of nerve stimulation (Dog B-181). Normal response occurs, not further increased by exogenous ACTH.

in a corticosteroid response which is not further increased by exogenous ACTH administration.

Figure 3 shows an experiment in which bilateral decortication was performed one week prior to adrenal vein cannulation. Variably high corticosteroid outputs were observed the day following cannulation, with immediate return to basal levels following anesthetization. Nerve stimulation resulted in a somewhat delayed but significant adrenal cortical response which was not further altered by exogenous ACTH. Another dog with chronic decortication yielded a similar protocol.

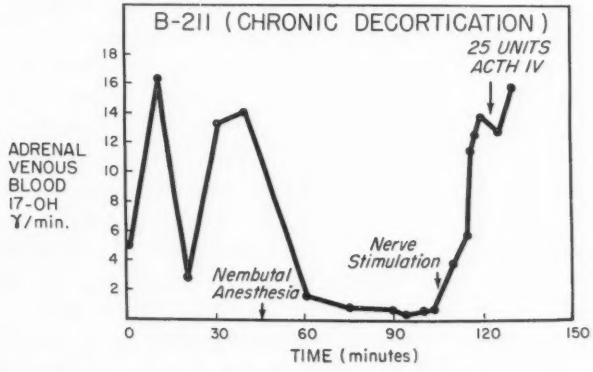

FIG. 3. Chronic decorticate dog (B-211) with same results as animal of Figure 2.

Most animals remained quiet prior to anesthesia, although some dogs demonstrated considerable motor activity and occasional low pitched barking. Adrenal venous catechol amine outputs were basal in the "resting" state in both normal and decorticate dogs, and a normal stimulatory response was obtained following nerve stimulation. In some experiments samples were collected in a dark room the day following decortication and this did not affect the results. Gross and microscopic examination of the brains revealed complete removal of the cerebral cortices, except for fragments of the temporal lobe in a few instances. Peripheral 17-hydroxycorticosteroid levels were elevated the day following decortication.

DISCUSSION

Indirect experimental evidence is available which suggests that the cerebral cortex inhibits ACTH release. The elevated blood corticosteroid levels observed following hypnosis (5) or situations productive of anxiety (6) could be a result of alteration or inhibition of impulses from the cerebral cortex which tonically inhibit ACTH release. It is equally possible that the cerebral cortex exerts a direct stimulatory effect on ACTH-releasing centers. Martin and co-workers (7) observed high adrenal venous corticosteroid outputs in dogs with previous bilateral removal of the amygdaloid nuclei. This was interpreted as meaning that the amygdala exerts an inhibiting influence on ACTH secretion. It is difficult to reconcile these findings with those of Mason *et al.* (8) who report a diminished peripheral blood 17-hydroxycorticosteroid response to conditioned emotional stimuli in monkeys following amygdalectomy.

Perhaps the most direct evidence that higher central nervous system areas inhibit ACTH release comes from the experiments of Slusher and Critchlow (9) who have shown that lesions in certain areas of the midbrain result in increased adrenal cortical function as determined by adrenal ascorbic acid depletion. The previous studies are subject to several different interpretations because they have either involved the use of indirect methods for determining adrenal cortical function or blood samples were taken during the immediate post-operative period.

Previous experiments from our laboratory have demonstraged that dogs with brains removed down to the pons and neurologically isolated pituitaries have high basal outputs of corticosteroids. A significant number of these animals respond to nerve stimulation (1) and vena caval constriction (10) with a further increase of corticosteroid output. These experiments have been interpreted as indicating the presence of another center, perhaps located in the hindbrain, which is involved in the control of ACTH release; other areas less likely to be envolved, such as the intestine, have not been ruled out. The postulated hindbrain center releases a neurohumor (hindbrain factor = HBF) which passes through the blood stream and causes ACTH release from the pituitary. The findings of the

present studies permit further speculation concerning the pathways involved in ACTH release.

Figure 4 outlines a concept for ACTH release mechanisms based on our present experimental evidence. Many possible alternative explanations have been considered, but do not appear to fit as well the presently available facts. According to the scheme in Figure 4, both the hypothalamus and hindbrain are capable of releasing neurohumors which pass through the systemic blood stream and cause ACTH release. The reticular formation is constantly bombarding the hypothalamus with stimulatory impulses for release of its neurohumor. The hindbrain is either stimulated

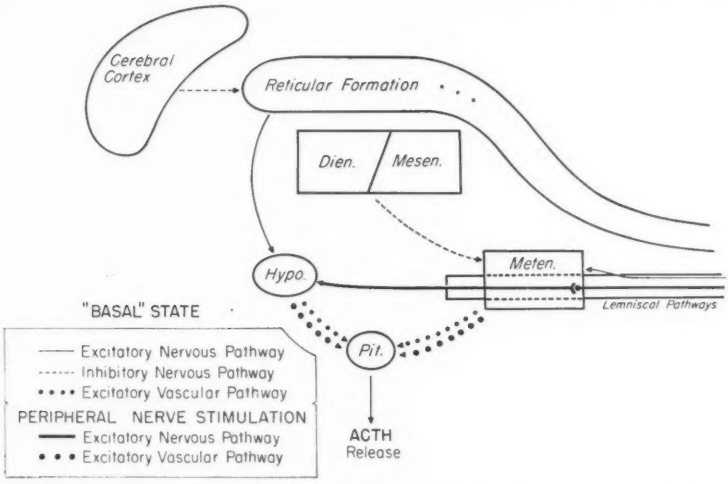

FIG. 4. Schematic representation of hypothesis for inter-relationships of areas concerned with ACTH release.

by tonic afferent impulses which ascend the spinal cord or it has a high degree of spontaneous activity. The cerebral cortex is thought of as exerting an inhibitory influence, either on the reticular formation or on pathways from the reticular formation to the hypothalamus. The hindbrain does not release HBF in the intact unstimulated animal because of inhibition by a portion of the diencephalon or mesencephalon.

The intact animal has "basal" outputs of corticosteroids because inhibition of hypothalamic and hindbrain stimulation is effectively carried out by the cerebral cortex and diencephalon-mesencephalon respectively. The intact animal reveals a normal response to peripheral nerve stimulation probably based on the passage of afferent nervous impulses up the spinal cord, through the hindbrain, to the hypothalamus. The interrelationships between hindbrain and hypothalamic ACTH-controlling centers have as yet not been elucidated. There are several possibilities in this regard, including the potentiation of hypothalamic corticotropin re-

leasing factor (CRF) with HBF, potentiation of HBF by a hypothalamic neurohumor, release of CRF by HBF, and no relationship between the two neurohumors. It is obvious, however, that inhibitory mechanisms are eliminated or overwhelmed by strong afferent stimulation.

Following decortication, the animals have elevated corticosteroid outputs. This is not due to surgical trauma in general, as shown by the presence of "basal" corticosteroid outputs on the day following adrenal vein cannulation and craniotomy without brain removal. The reticular formation is uninhibited, and the hypothalamus is in a state of constant activity with regard to release of CRF. The hindbrain center remains inhibited. Following Nembutal anesthesia, "basal" levels are observed in the decorticate animal. French and co-workers (11) have demonstrated that the principal locus of action of Nembutal is the reticular formation, and there is experimental evidence for decreased electrical potentials from this area following Nembutalization. The decorticate dog remains normally responsive to afferent nerve stimulation following anesthesia because the ACTH-releasing centers are intact. Corticosteroid outputs following nerve stimulation are the same in normal and decorticate anesthetized animals.

Dogs with brain removal down to the pons and isolated pituitaries demonstrate elevated corticosteroid outputs because of removal of diencephalic or mesencephalic areas which inhibit the hindbrain. Either because of tonic afferent impulses, or a high rate of spontaneous activity, the hindbrain releases HBF and ACTH release takes place. Recent studies have demonstrated that Nembutal anesthesia does not lead to depression of the elevated corticosteroid outputs which occur in dogs with isolated pituitaries. Anesthetic doses of Nembutal have little effect on peripheral nerve fiber conduction and less effect on most hindbrain areas than on the reticular formation. Accordingly, HBF continues to be released following Nembutalization.

There are objections to most alternative explanations. It is possible that the cerebral cortex exerts its inhibition through direct nervous pathways to the hypothalamus. According to this concept, the elevated levels of corticosteroids in decorticate dogs would be due to a high spontaneous rate of activity of the hypothalamus. Nembutalization, however, results in depression of these elevated levels and this would imply a direct action of Nembutal on the hypothalamus. Although there is experimental evidence for hypothalamic depression following anesthesia (12), this effect is less marked than that on the reticular formation, and the Nembutalized hypothalamus might not be expected to give a normal maximal response to nerve stimulation. It is possible that the elevated corticosteroid outputs following decortication can be explained on some other basis than removal of inhibition. Short term metabolic studies on these animals have revealed normal electrolytes on the days of experimentation, good urine output, and relatively normal blood pressures and pulses. The finding of increased

peripheral blood levels of corticosteroids rules out the possibility of hypothalamic stimulation due to low blood steroid levels. It is possible that the decorticate dog is hypersensitive to exogenous stimuli and attempts were made in these experiments to keep such factors to a minimum. The experimental results were unaltered by environmental condition changes, or variations in motor activity of the animals themselves. It is difficult to explain the elevated corticosteroid outputs in the decorticate animals on any basis other than removal of inhibition of lower centers concerned with ACTH release. Experiments involving partial decortication are currently being carried out in order to anatomically localize areas responsible for inhibition.

There are several possible interpretations for the results following Nembutal anesthesia. Nembutal may act primarily on other areas of the brain than the reticular formation, although the evidence is convincing that the reticular formation is depressed following Nembutal anesthesia to a degree which exceeds that of the rest of the central nervous system (11). It is possible that Nembutal acts directly on the pituitary, but normal response to nerve stimulation makes this possibility unlikely. Nembutal may act directly on the adrenal cortex and this has not been ruled out by the data of this report. It is possible that the amount of endogenous ACTH which results in an elevated "basal" adrenal cortical response in decorticate dogs is much smaller than that amount which is released following nerve stimulation. According to this concept, the adrenal following Nembutal loses sensitivity to the small quantity of ACTH which previously has been endogenously released. Therefore, although the adrenal is depressed, a significant response to the additional endogenous ACTH released in response to nerve trauma occurs. Failure to observe post-anesthesia depression of elevated "resting" corticosteroid levels in dogs with brain removal is evidence against a significant direct action of Nembutal on the adrenal.

REFERENCES

1. Egdahl, R. H.: *Endocrinology* **66:** 200. 1960.
2. Hume, D. M. and D. H. Nelson: *Surg. Forum* **6:** 568. 1955.
3. Nelson, D. H. and L. T. Samuels: *J. Clin. Endocrinol. and Metab.* **12:** 519. 1952.
4. Aranow, L., F. A. Howard and D. wolff: *J. Pharm. Exp. Ther.* **116:** 1. 1956.
5. Persky, H., H. J. Grosz, J. A. Norton and M. McMurtry: *J. Clin. Endocrinol. and Metab.* **19:** 700. 1959.
6. Euler, U. S. V., C. A. Gemzell and L. Levi: *Acta Endocrinologica* **30:** 567. 1959.
7. Martin, J., E. Endroczi and G. Bata: *Acta Phyio. Hung.* **14:** 131. 1958.
8. Mason, J. W.: *Am. J. Physiol.* **196:** 44. 1959.
9. Slusher, M. A. and V. Critchlow: *Proc. Soc. Exper. Biol. & Med.* **101:** 497. 1959.
10. Egdahl, R. H.: Corticosteroid secretion following caval constriction in dogs with isolated pituitaries. In Press, *Endocrinology.*
11. French, J. D., M. Verzeano and H. W. Magoun: *Arch. Neurol. Psychiat.* **69:** 519. 1953.
12. Masserman, J. H.: *Arch. Neurol. Psychiat.* **37:** 617. 1937.

EFFECT OF GRADED DOSES OF PROPYLTHIOURACIL ON BIOSYNTHESIS OF THYROID HORMONES[1]

SHIRO IINO,[2] TAKASHI YAMADA[2] AND MONTE A. GREER

Division of Endocrinology, Department of Medicine, University of Oregon Medical School, Portland, Oregon

ABSTRACT

Graded doses of PTU (0.001–180 mg.) were administered subcutaneously once daily to rats for three days. I^{131} was given immediately following the last dose of PTU; twenty-four hours later the thyroids were removed. Relative amounts of DIT and of T3+T4 decreased with increasing doses of PTU. No DIT nor T3+T4 were found with doses greater than 0.1 mg. The relative amount of MIT increased with the amount of PTU, attained its maximum at a dose of 5 mg., then declined. Even with 180 mg. of PTU, MIT formation was not blocked completely. The absolute amount of radioiodide declined sharply with doses greater than 0.01 mg. Formation of DIT from MIT is thus inhibited by very small doses of PTU, whereas organic binding of iodide into MIT is not completely blocked even with very large doses. Coupling to form iodinated thyronines is depressed by smaller amounts of PTU than the other biosynthetic mechanisms studied.

RECENTLY it has been established that propylthiouracil (PTU) inhibits not only the initial monoiodination of tyrosine but also both the diiodination of tyrosyl radicles and coupling to form triiodothyronine and thyroxine (1, 2). However, the effect of large doses of PTU on the biosynthesis of thyroid hormones has not been studied in detail. Therefore, we considered it of interest to investigate this problem in the rat.

MATERIALS AND METHODS

Male Holtzman rats weighing approximately 250 gm. were employed in experiments performed at three different times. Following seven days of low iodine diet, graded doses (0.001, 0.01, 0.1, 1.0, 5, 30, 60, 90 and 180 mg.) of PTU were administered subcutaneously once daily for three days. Three or four animals were used at each dose level. Twenty to 100 μc of I^{131} were given intraperitoneally immediately following the last dose of PTU. Twenty-four hours later, the animals were killed and the radioactivity of the thyroid glands was determined with a well-type scintillation counter. The thyroids, pooled in some instances, were homogenized and hydrolyzed with three ml. of 0.5% pancreatin (Viokase 4x) solution at pH 8.6 at 39° C for 24 to 72 hours. Several drops of toluene were added to prevent putrefaction. Portions of the digests containing 0.025 μC were subjected to ascending paper chromatography in butanol-acetic acid-water (4:1:5) for 16 hours. The radioactivity of each chromatogram was analyzed with a continuous

Received June 28, 1960.

[1] Supported by USPHS Grants A-1447 and A-2503.

[2] Trainee of National Institute of Arthritis and Metabolic Diseases.

automatic strip counter and the areas under the curves were measured by planimeter. Each compound was identified by comparing its Rf value with that of a standard. Since the triiodothyronine (T3) fraction runs very close to the thyroxine (T4) fraction in this solvent system, it is quite difficult to distinguish T3 from T4. Both fractions were therefore calculated as one and shown as T3+T4 in this experiment.

RESULTS

Effect of graded doses of PTU on thyroidal I^{131} uptake

The I^{131} uptake decreased strikingly at a dose level of 0.1 mg. and was depressed below 1% when 1 mg. or more was employed (Fig. 1).

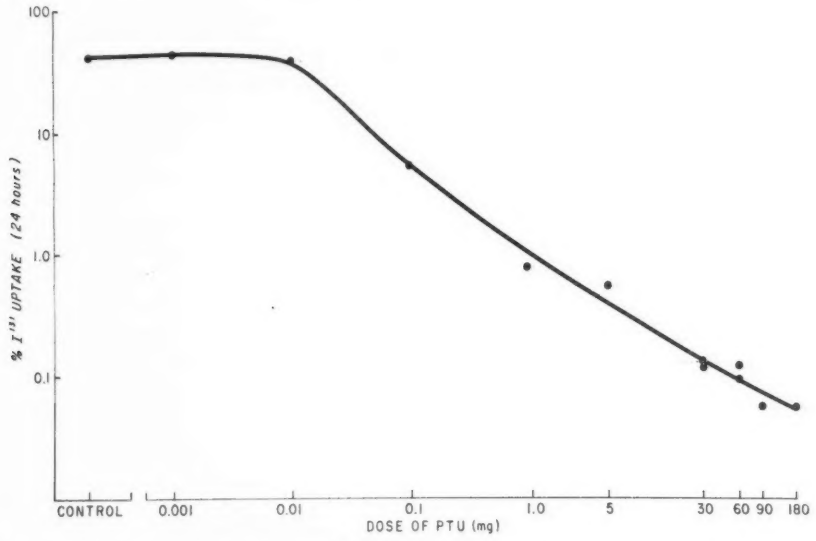

FIG. 1. Effect of graded doses of PTU on thyroidal I^{131} uptake. Log-log plot. Each point represents mean of 3–4 animals.

Relationship between graded doses of PTU and the relative amounts of radioiodinated amino acids and inorganic iodide in the thyroid

The relative amount of T3+T4 decreased to approximately half of the control value at a dose of 0.001 mg., then gradually declined further with increasing amounts of PTU (Fig. 2). Diiodotyrosine (DIT) was within or above the control range at dose levels up to 0.01 mg., then decreased. No DIT or T3+T4 were found when 1 mg. or more of PTU was given. Monoiodotyrosine (MIT) increased with increasing amounts of PTU, attained its maximum at 5 mg., then declined. Even with 90 mg. PTU, MIT still accounted for more than 10% of the total radioactivity. No MIT was found in one of the animals treated with 180 mg. of PTU and the average MIT content of this group was less than 5% of total thyroidal radio-

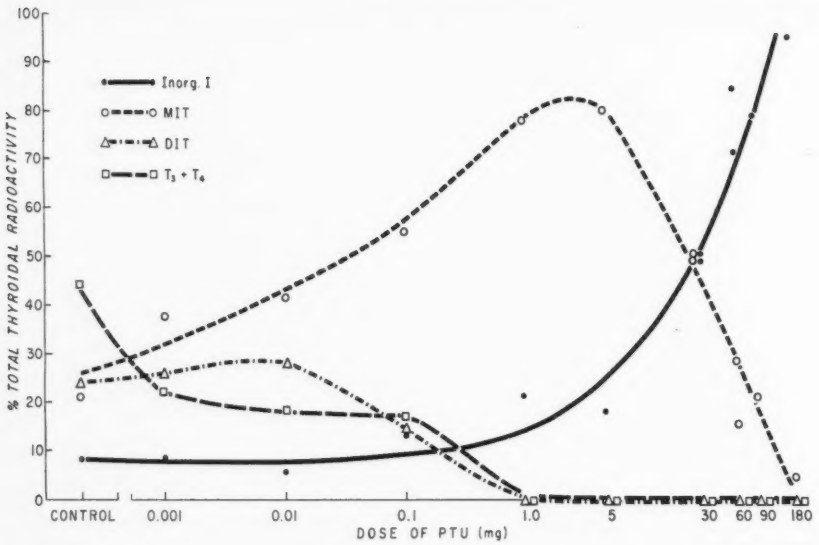

FIG. 2. Relative amounts of radioiodinated amino acids in the thyroid after administration of graded doses of PTU. Semi-log plot. Each point represents mean of 3–4 animals.

activity. The relative amount of inorganic iodide increased with the dose of PTU, approaching 100% at 180 mg. Representative chromatograms of this experiment are illustrated in Fig. 3 and 4.

Relationship between graded doses of PTU and the absolute amounts of radioiodinated amino acids and inorganic iodide in the thyroid

The absolute amounts of T_3+T_4, as measured in terms of percent of the I^{131} administered, decreased to approximately half of the control value when 0.001 mg. PTU was given, then declined sharply with amounts greater than 0.01 mg. (Fig. 5). The amount of DIT when less than 0.1 mg. was administered was within the control range, then declined rapidly with further increments of PTU. The amount of MIT increased initially with the dose of PTU, attained a maximum of twice the control value at a dose of 0.01 mg., then decreased. The amount of inorganic iodide declined strikingly with doses greater than 0.01 mg. and apparently reached a plateau at a level 1/50 that of the iodide concentration of the controls (3% compared to 0.06%).

Effect of graded doses of PTU on the DIT/MIT ratio

The DIT/MIT ratio decreased as the amount of PTU increased, reaching 0 at a dose of 1.0 mg., at which point DIT disappeared.

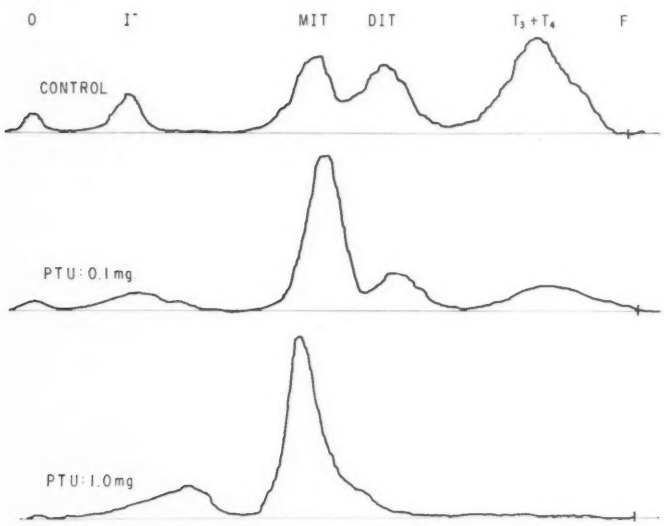

FIG. 3. Scans of radioactivity of chromatograms of thyroid hydrolysates. O = origin, I^- = iodide, MIT = monoiodotyrosine, DIT = diiodotyrosine, T3 + T4 = triiodothyronine + thyroxine, F = front.

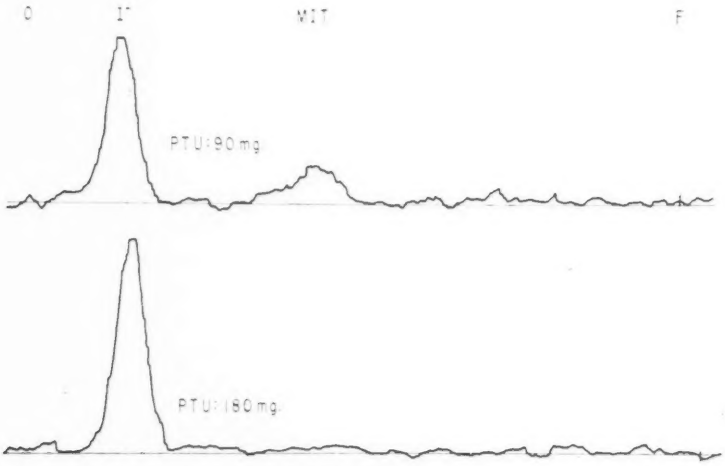

FIG. 4. Legend as in Fig. 3. The one out of three animals receiving 180 mg. PTU in which no monoiodotyrosine was found is depicted.

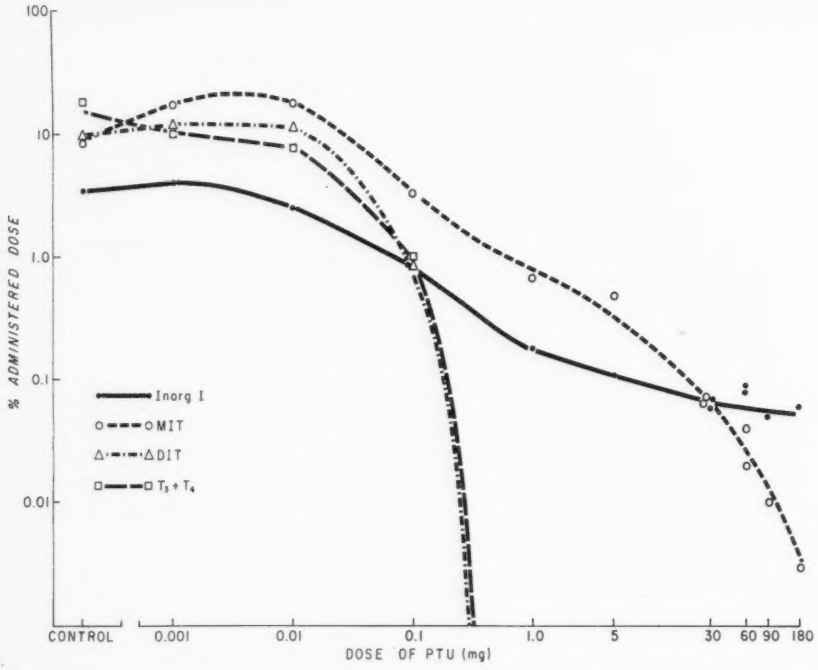

FIG. 5. "Absolute" amounts of radioiodinated amino acids and iodide, expressed as percent of the administered dose of I^{131}, after graded doses of PTU. Log-log plot. Each point represents mean of 3–4 animals.

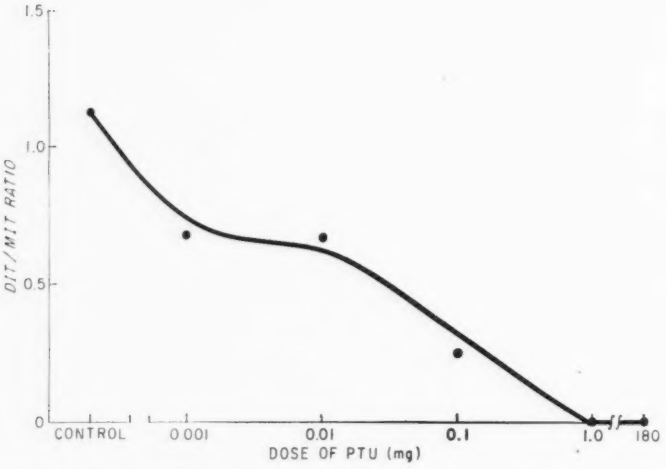

FIG. 6. Semi-log plot. Each point represents mean of 3–4 animals.

DISCUSSION

The results indicate that the formation of DIT from MIT is inhibited by very small doses of PTU, whereas organic binding of iodide into MIT is not completely blocked even with relatively large doses. This finding confirms the studies of Slingerland *et al.* (1) and of Richards and Ingbar (2). It should be noted that, even with 180 mg. of PTU, MIT formation cannot be blocked completely. The fact that T3+T4 was decreased by half with 0.001 mg. of PTU, whereas DIT was still at the control level, indicates that PTU more readily inhibited the coupling of iodinated tyrosines to form iodothyronines than other processes, as suggested by Richards and Ingbar (2). However, it is also probable that formation of DIT from MIT was partially blocked at this level because the DIT/MIT ratio decreased. Accordingly, the processes in order of decreasing sensitivity to PTU are T3+T4 formation, DIT formation and MIT formation.

Antithyroid drugs of the thioureylene group supposedly inhibit the formation of organic iodine compounds while inorganic iodide can still enter the thyroid and accumulate there in normal or even increased amounts (3, 4). In our study, the absolute amount of inorganic iodide in the thyroid gland decreased strikingly with increasing doses of PTU. It is possible that part of the iodinated organic compounds may have been deiodinated *in vivo* (5) or by the pancreatin hydrolysis *in vitro* and thus have contributed to the higher iodide values found with small doses of PTU. According to VanderLaan and Bissell (6) and Taurog *et al.* (10), the thyroid gland of the rat under the influence of PTU readily takes up injected iodide, but is able to retain this iodide for only a short time. Since our determinations were made 24 hours after radioiodine injection, it seems likely that a large part of the inorganic iodide accumulated by the thyroid gland had already been lost by this time. Brown (8) has shown that PTU increases the renal clearance of iodide in the rat. It is probable that such a mechanism explains our findings, the exchangeable iodide held in the thyroid gland thereby being more rapidly depleted.

Further studies on the effect of PTU on I^{131} accumulation and discharge by the thyroid gland (7) have shown that the iodide accumulated under the influence of PTU was quickly lost, whereas in the control group the reverse was true, iodide content actually increasing with time.

The possibility that a low intrathyroidal concentration of iodine might contribute to the shifts in the proportions of iodinated compounds in the gland has been studied by Slingerland *et al.* (1) and by Richards and Ingbar (2). These authors used thiocyanate and perchlorate, respectively, and found that they did not produce the same modifications as PTU.

So far as we are aware, formation of monoiodotyrosine has not been reported previously when large doses of propylthiouracil are employed. Pitt-Rivers *et al.* (9) found that the subsequent administration of perchlor-

ate to rats treated with 5 mg. of PTU and given 0.1% thiouracil in the drinking water would result in some MIT formation. No MIT was formed if perchlorate was not given, however. One possible explanation for the discrepancy in results is that their animals were killed two hours after radioiodine administration whereas ours were killed at 24 hours. This explanation seems inadequate, however, since other studies in our laboratory (7) have shown that one hour after the administration of 30 mg. PTU and I^{131}, using the three-day technique described herein, 4.7% of the intrathyroidal radioactivity is present as MIT while by three hours this has risen to 20%. Pitt-Rivers has recently found that perchlorate will not increase PBI^{131} in PTU-treated rats (11), in contrast to her earlier results.

REFERENCES

1. Slingerland, D. W., D. E. Graham, R. K. Josephs, P. F. Mulvey, Jr., A. P. Trakas and E. Yamazaki: *Endocrinology* **65**: 178. 1959.
2. Richards, J. B. and S. H. Ingbar: *Endocrinology* **65**: 198. 1959.
3. Astwood, E. B.: *Harvey Lectures* **40**: 195. 1944–45.
4. VanderLaan, J. E. and W. P. VanderLaan: *Endocrinology* **40**: 403, 1947.
5. Roche, J. and R. Michel: *Physiol. Rev.* **35**: 583. 1955.
6. VanderLaan, W. P. and A. Bissell: *Endocrinology* **39**: 159. 1946.
7. Iino, S.: Unpublished data.
8. Brown, J.: *Endocrinology* **58**: 68. 1956.
9. Pitt-Rivers, R., V. A. Galton and N. S. Halmi: *Endocrinology* **63**: 699. 1958.
10. Taurog, A., I. L. Chaikoff and D. D. Feller: *J. Biol. Chem.* **171**: 189. 1947.
11. Pitt-Rivers, R.: Ann. N. Y. Acad. Sc. **86**: 362. 1960.

THE LIPOLYTIC RESPONSE TO CORTICOTROPIN[1,2]

C. H. HOLLENBERG[3], M. S. RABEN AND E. B. ASTWOOD

Pratt Clinic-New England Center Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts

ABSTRACT

Exposure of isolated rat adipose tissue to small concentrations of corticotropin (.001 μg/ml.) caused accumulation of free fatty acid in tissue and medium, a finding previously reported by others. The effect of corticotropin was inhibited by glucose and insulin, presumably due to promotion of esterification of the free acids. Corticotropin was also found to be active *in vivo*; intravenous administration of this hormone to acutely adrenalectomized rats produced rapid increase of free fatty acids in adipose tissue and plasma. The lipolytic activity of homogenates of rat adipose tissue was increased by brief exposure of the intact tissue to corticotropin and epinephrine. The activity affected by ACTH differed from that of lipoprotein lipase in pH optimum. These results suggest that corticotropin may produce its adipokinetic effect by directly activating a lipase in adipose tissue.

A POSSIBLE role of corticotropin in the regulation of fat mobilization was suggested by the studies of Rosenberg (1) which showed that corticotropin increased liver fat in the mouse by an extraadrenal action, and more recently, by the findings of White and Engel (2) that the hormone stimulated the production of free fatty acids by isolated rat adipose tissue.

The effects of corticotropin on free fatty acids were studied in further detail in the study to be reported here; the increased fatty acid production caused by either corticotropin or epinephrine was shown to be associated with increased lipolytic enzyme activity in the adipose tissue.

METHODS

Male albino rats weighing 150 to 200 gm. and obtained from Charles River Laboratories, Boston, were used throughout the study. The hormone preparations used were pig corticotropin prepared by the oxycel purification method (3), human growth hormone prepared by the acetic acid extraction procedure (4) and commercial preparations of epinephrine (Parke, Davis) and crystalline insulin (Lilly). The human albumin used in the *in vitro* experiments was pretreated to remove bound fatty acid (5). The measurement of free fatty acids was made by the method of Dole (6).

Received June 30, 1960.

[1] Presented at the annual meeting of The Endocrine Society, Miami Beach, Florida, June, 1960.

[2] This study was supported by Grants A-1567 and A-612 from the National Institutes of Health, Bethesda, Maryland.

[3] Medical Research Fellow, National Research Council, Canada. Present address: McGill University Clinic, Montreal General Hospital, Montreal, Quebec, Canada.

In experiments with surviving adipose tissue, pieces of rat epididymal fat were incubated in 1 ml. of Krebs-Ringer phosphate buffer containing 5% human albumin (pH 7.4). Incubation was carried out for 3 hours (unless otherwise indicated) at 37° C in air, in a Dubnoff shaker, and the free fatty acid content of tissue and medium estimated at the end of incubation. The results were expressed as μEq. of free fatty acid per gram of tissue per unit time of incubation. The samples of tissue for the control and experimental flasks were always obtained from the same animal.

In experiments in which the activity of lipolytic enzyme was measured the fat tissue was homogenized in phosphate buffer and an aliquot containing the equivalent of about 50 mg. of the original tissue was added to the substrate mixture. The latter was a coconut oil emulsion (Ediol-Schenlabs) in a concentration of 0.5% diluted in phosphate buffer containing 5% human albumin. In experiments in which the effect of variation in pH on lipolytic activity was studied, Tris buffer was added to the substrate mixture in a final concentration of 0.1 M. The homogenate-substrate mixture in a volume of 3 ml. was incubated at 37° C under air for 30 minutes. Lipolysis was determined by change in the free fatty acid content of the mixture during incubation and expressed as μEq. of free fatty acid produced per gram of tissue per 30 minutes. Lipolytic activity was assayed at several hydrogen ion concentrations and pH change during incubation rarely exceeded 0.2 pH units.

RESULTS

Sensitivity of surviving rat adipose tissue to corticotropin

The effect of corticotropin on the quantity of free fatty acid found after incubation of surviving adipose tissue from fed and fasted animals is shown in Figure 1. In agreement with the data of Engel and White (7) minute concentrations of corticotropin produced a distinct effect; tissue from fasted animals responded to .001 μg/ml., and fed tissue to .003 μg/ml., the smallest amount tested in this instance. Fasted tissue produced more free

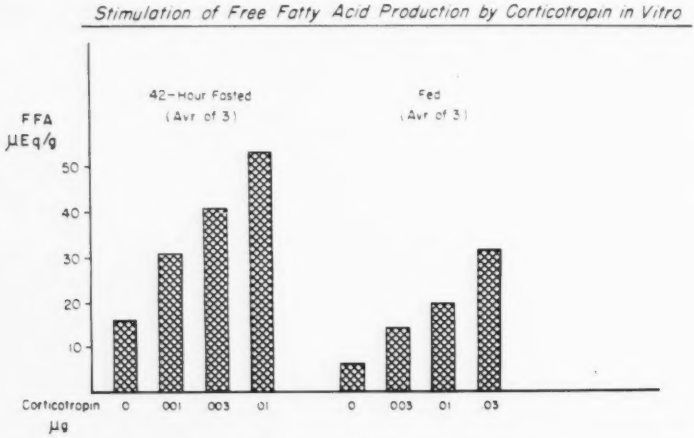

FIG. 1. Stimulation of Free Fatty Acid Production by Corticotropin *in Vitro*. The tissues were incubated for three hours and the results expressed as μEq. of free fatty acid per gram of tissue per three hours of incubation.

fatty acid than did fed in response to the same concentrations of corticotropin. As esterification of fatty acids has been shown to be enhanced by augmentation of carbohydrate metabolism in adipose tissue (8, 9), reduction in the rate of esterification of the free acid in the fasted tissues may have been responsible for the greater response when this tissue was used. With either fed or fasted tissue progressively larger doses of corticotropin produced progressively larger effects; a maximum response was usually observed with concentrations in the range of 1.0 to 10.0 μg/ml.

Figure 2 shows the effect of three pituitary preparations containing corticotropin on the free fatty acid response of isolated fat tissue. As the

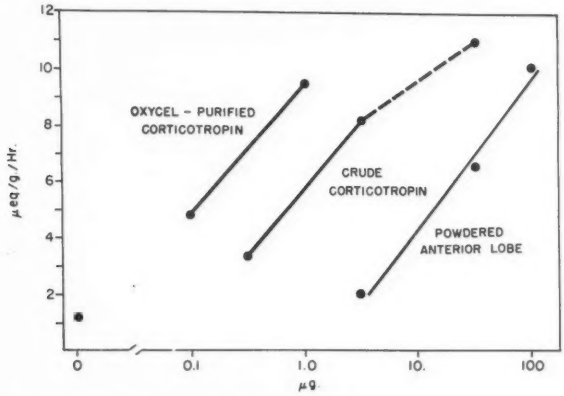

FIG. 2. Effect of Anterior Pituitary Powder, Crude and Purified Corticotropin on Free Fatty Acid Response *in Vitro*. The anterior pituitary powder (acetone-dried pig anterior lobe) was tested as a suspension in the medium, the crude ACTH was prepared from a glacial acetic acid extract of pig anterior lobe and the purified corticotropin was concentrated from the crude by adsorption on oxycellulose. The tissues were incubated for three hours and the results expressed as μEq. of free fatty acid per gram of tissue per hour of incubation.

corticotropin concentration in the preparations was increased by purification smaller doses were necessary to produce equivalent responses. Although purification with oxycel increased potency only ten-fold, it is probable that most of the activity of the anterior pituitary powder was due to its corticotropin content.

The effect of glucose and insulin on the response of surviving adipose tissue to corticotropin

The inhibition by carbohydrate of the response of free fatty acids to fasting has been observed both in the intact animal and *in vitro* (6, 10, 11). Table 1 shows the inhibitory action of glucose and insulin added *in vitro* on the response of adipose tissue to corticotropin. With a concentration of 200 mg.% glucose in the medium, 10 μU. of insulin produced distinct

TABLE 1. THE EFFECT OF GLUCOSE AND INSULIN ON THE FREE FATTY ACID RESPONSE TO CORTICOTROPIN *in vitro**

Rat No.	ACTH	Insulin					
		0	1 μU./ml.	10 μU./ml.	100 μU./ml.	1 mU./ml.	10 mU./ml.
1	1 γ/ml.	7.8	5.3	4.1	2.0	1.7	1.7
2	1 γ	7.1	9.3	3.1	1.7	1.8	1.9
3	1 γ	16.8	9.1	10.0			
4	1 γ	9.5	6.6	4.6			
5	10 γ/ml.	14.3	13.3	11.6	5.4		
6	10 γ	16.0	14.8	12.0	6.6		

* The tissue was incubated for three hours and the results are expressed as μEq. of free fatty acid per gram of tissue per hour of incubation.
Fat from fed animals was used in all experiments and 200 mg.% glucose was present in all flasks.

inhibition of the fatty acid response to either 1 or 10 μg/ml. of corticotropin. The response to 1 μU. of insulin was less, especially when larger concentrations of corticotropin were used. This effect of glucose and insulin is most likely due to stimulation of esterification of free fatty acids in this tissue.

The effect of corticotropin on free fatty acids in vivo

In vivo studies in man and dogs have failed to show a clear effect of corticotropin on the free fatty acids of plasma (12). As rat adipose tissue is extremely sensitive to corticotropin added *in vitro*, its effect on the free fatty acids of the intact rat was studied. In these experiments 0.1 to 1.0 mg. of corticotropin was given intravenously to acutely adrenalectomized rats, while a control group was given saline. Samples of adipose tissue and of aortic blood were taken, on the average 40 minutes after the injection, for measurement of free fatty acids. The fat tissue was incubated in buffer-albumin for three hours after which the fatty acid content of tissue and medium was measured. The results of this study shown in Table 2 indicate that the administration of corticotropin resulted in a significant increase in the free fatty acids of adipose tissue and of plasma. Although these results indicate that the *in vitro* effect of corticotropin can be reproduced *in vivo*,

TABLE 2. FFA AFTER INTRAVENOUS CORTICOTROPIN IN ADRENALECTOMIZED RATS

No. rats	Corticotropin, mg.	Time after corticotropin minutes	Incubated adipose tissue FFA, μEq./gm./3 hrs.	Plasma FFA, μEq./L.
10	0	—	11.7 ± 1.2	1126 ± 166
12	0.1–1.0	39 (13 to 70)	19.4 ± 1.6*	2186 ± 246

± = S.E.
* 11 animals used.
Statistical Analysis—adipose tissue FFA-control vs. corticotropin $P < .01$.
plasma FFA-control vs. corticotropin $P < .01$.

they do not establish the physiological significance of corticotropin as an adipokinetic agent since very large amounts of hormone were used to evoke this effect.

The effect of corticotropin on a lipolytic enzyme of adipose tissue

One mechanism by which corticotropin could increase the production of free fatty acids by adipose tissue would be by activation of a lipase in this tissue. To explore this possibility paired pieces of rat epididymal fat from fed rats and from rats fasted for 24 hours were incubated with and without 20 μg/ml. of corticotropin in phosphate buffer for 15 minutes at 37° C. Earlier studies had indicated that exposure of fat tissue to this dose of corticotropin over this brief interval was sufficient to produce a free fatty acid response. A smaller number of experiments with similar concentrations of epinephrine and human growth hormone were performed using fat from fed animals. Following incubation the tissues were homogenized and the lipolytic activity assayed at pH 7.0 to 7.3 as described above. The results of these experiments are shown in Figure 3.

Exposure of intact tissues from fed or fasted rats to 20 μg/ml. of corticotropin invariably caused an increase in the lipolytic activity measured after the tissue had been homogenized, the mean increase with fed or

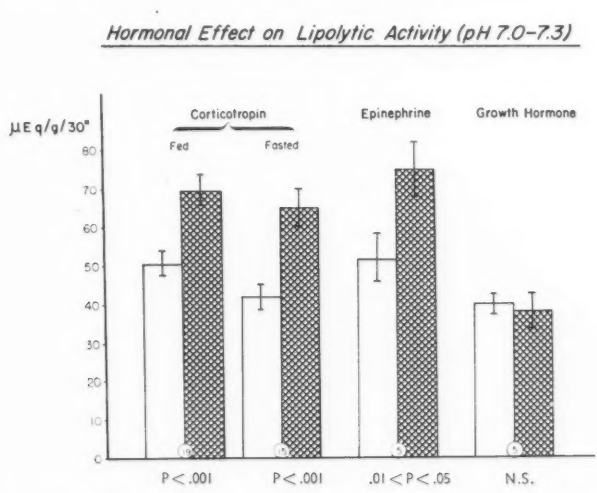

FIG. 3. Hormonal Effects on Lipolytic Activity of Rat Adipose Tissue (pH 7.0–7.3). 0.3 to 0.5 ml. of homogenate was added to 0.3 ml. of 5% coconut oil, 0.75 ml. of 20% human albumin and to sufficient Krebs-Ringer phosphate buffer to bring volume to 3.0 ml. In some experiments 0.7 ml. of Tris Buffer (0.4 M) replaced part of the phosphate buffer. The initial pH in all experiments varied from 7.0 to 7.3. The brackets indicate S.E., the number of experiments are indicated in the circles and the P values are given at the foot of the columns (N.S. = not significant). Lipolysis is expressed as μEq. of free fatty acid produced per gram of tissue per 30 minutes of incubation.

fasted tissue being about 40%. One μg/ml. of corticotropin gave an effect of similar magnitude while 0.1 μg/ml. produced a significant but smaller increment. Corticotropin was without effect when added after the tissue had been homogenized.

Twenty micrograms per ml. of epinephrine caused an increase in lipolytic activity of the same magnitude as that produced by a similar concentration of corticotropin, while growth hormone in this concentration was inactive. While the administration of small doses of growth hormone *in vivo* is effective in increasing the concentration of free fatty acid in plasma in both man and dog (12) and in increasing the production of free fatty acid by

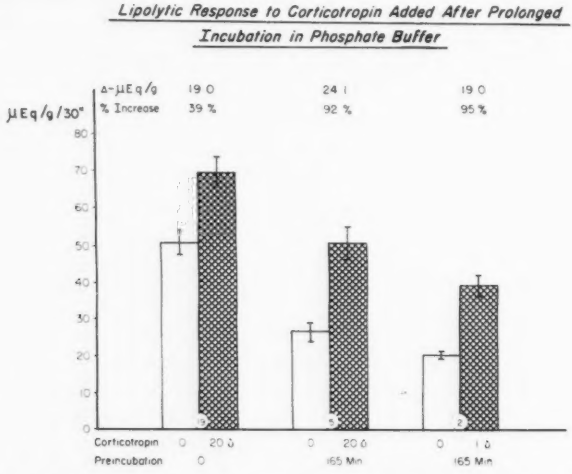

FIG. 4. Lipolytic Response to Corticotropin Added after Prolonged Incubation in Phosphate Buffer. The composition of the homogenate-substrate mixture and the expression of results are the same as described in the Legend to Figure 3.

adipose tissue from hypophysectomized rats (13, 14), *in vitro* effects of growth hormone have been found only with large amounts of the hormone and may be attributable to traces of corticotropin or other factors in the preparation.

Figure 4 shows the effect on lipolytic activity of prolonged incubation of intact tissue in phosphate buffer before homogenizing. Paired pieces of epididymal fat were incubated in buffer for 165 minutes, corticotropin was then added to one flask and incubation continued for an additional 15 minutes. The tissues were then homogenized and lipolytic activity assayed at pH 7.0 to 7.3. While preincubation lowered the activity of the tissues markedly, the magnitude of the response to corticotropin was not altered and thus the per cent increase in activity induced by the hormone was greater with the preincubated tissue. These experiments suggest that the

magnitude of the effect from corticotropin is not directly dependent on the basal level of lipolytic activity of the tissue.

Korn has found that extracts of rat heart (15) and rat and chicken adipose tissue (16, 17) contain a lipolytic enzyme, the optimum substrate for which is a lipoprotein-triglyceride complex and which was therefore termed lipoprotein lipase. To explore the possible relationship between the activity of this enzyme and that induced by corticotropin a study was made of the effect of pH on these activities. Aliquots of homogenates of control and corticotropin-treated tissues were added to coconut oil emulsions diluted in a mixture of Krebs-Ringer phosphate and Tris buffers at pH's 6.1, 7.0, 7.9. To explore the effect of pH on the activity of lipoprotein lipase in the homogenate system, use was made of the fact that fasting reduces the activity of lipoprotein lipase in rat adipose tissue (5, 18, 19). Thus aliquots of fat homogenates from fed and 24-hour fasted rats were treated in a fashion identical to that used in the experiments with corticotropin except for the addition of small amounts of serum, and hence lipoprotein, to the coconut oil mixture. The results of these experiments are shown in Figure 5.

Homogenates from fasting animals were less active than those from fed only at the highest pH tested, 7.9, indicating that lipoprotein lipase, the activity of which is reduced by fasting has more activity at pH 7.9 than at 7.0 in the homogenate system. This result is consistent with the observation by Korn that the activity of lipoprotein lipase prepared from chicken adipose tissue increases with increasing pH up to 8.5 (17). In contrast to this finding the increase in activity induced by corticotropin expressed either in absolute terms as in Figure 5, or as a percentage increase over the control, was greatest at pH 7.0. When the corticotropin-induced activity was assayed using the serum-triglyceride substrate, the same pH optimum was found while the increment in activity with this substrate was at least as great as and in some instances was greater than with triglyceride alone.

Of the various inhibitors of the corticotropin-induced lipolytic activity studied the most effective was sodium fluoride. In these experiments paired pieces of epididymal fat were incubated with and without 20 μg/ml. of corticotropin and then homogenized as previously described. One aliquot of each homogenate was then added to a coconut oil preparation (pH, 7.0) containing .027 M sodium fluoride and a second aliquot of the same homogenate added to substrate without sodium fluoride. Assay of lipolytic activity was performed as before. The results of these studies are shown on the left side of Figure 6. The effect of sodium fluoride was to inhibit by approximately 45% the lipolytic activity of the control homogenates and to reduce by 80% the activity induced by corticotropin. When sodium fluoride was incubated with intact adipose tissue in the presence of corticotropin, it inhibited the response to this hormone (Right side, Figure 6). It

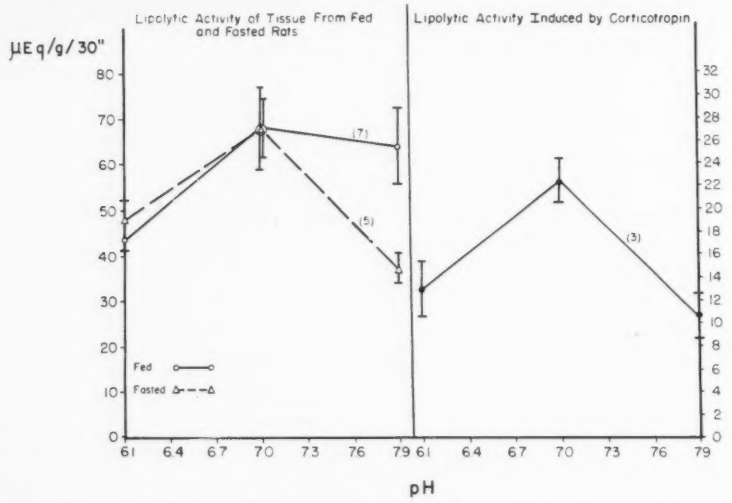

FIG. 5. Effect of pH on the Lipolytic Activity of Rat Adipose Tissue. *Left*—the medium consisted of 0.3 ml. of 5% coconut oil, 0.3 ml. of fresh human serum, 0.7 ml. of 0.4 M Tris buffer, 0.68 ml. of 20% albumin and 0.72 ml. of phosphate buffer. This mixture was incubated at 37° C for 30 minutes, pH was then adjusted and 0.3 ml. of homogenate added. *Right*—the medium consisted of 0.3 ml. of 5% coconut oil, 0.75 ml. of 0.4 M Tris buffer, 0.70 ml. of 20% albumin and 0.95 ml. of phosphate buffer. After adjustment of pH, 0.3 ml. of homogenate was added. The values plotted represent the difference between the lipolytic activity of control and corticotropin-treated tissues expressed as μEq. per gram of tissue per 30 minutes of incubation.

The S.E. and number of experiments are shown.

would appear reasonable to relate this latter effect of sodium fluoride to its inhibitory action on the lipolytic enzyme affected by corticotropin.

DISCUSSION

The findings in these experiments suggest that corticotropin and epinephrine augment free fatty acid accumulation in the rat by activating a lipase in adipose tissue. These findings are similar to those recently reported from other laboratories (20, 21).

The rapid activation of lipase produced by epinephrine and corticotropin is in keeping with the *in vivo* effects of these hormones. Epinephrine has been shown to cause a rapid increase in the free fatty acids of plasma in several species (6, 10, 12) and corticotropin has now been found to behave similarly in the rat. It is of interest that growth hormone produces a rise in plasma fatty acids more slowly (in man only after 2 hours) and that this hormone in a dose of 20 μg/ml. failed to produce a prompt effect on the lipolytic activity of adipose tissue. It is possible that these differences are due to the production by growth hormone of release of free fatty acid by a mechanism different from that affected by ACTH and epinephrine.

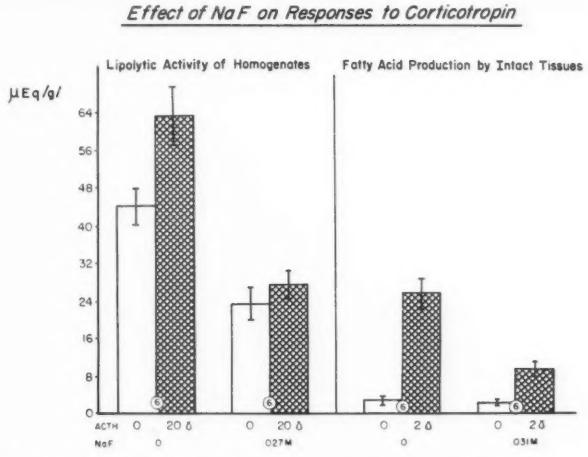

FIG. 6. Effect of Sodium Fluoride on Responses to Corticotropin. *Left*—0.5 ml. of homogenate was added to 0.3 ml. of 5% coconut oil, 0.75 ml. of 20% albumin and 1.45 ml. of phosphate buffer, the medium having been preadjusted to pH 7.0. Lipolysis is expressed as μEq. of fatty acid produced per gram of tissue per 30 minutes. *Right*—medium consisted of 1 ml. of phosphate buffer with 5% albumin and incubation was carried out for 1 hour. Results are expressed as μEq. of free fatty acid per gram of tissue per hour.

The lipolytic activity induced by corticotropin differs from that of lipoprotein lipase in pH optimum, and it is not reduced by fasting, a condition which reduces lipoprotein lipase activity. Thus the enzyme affected by corticotropin may be distinct from lipoprotein lipase but it must be further characterized before a definitive statement concerning this distinction can be made. An additional problem that must await further study is the relationship between the lipase stimulated by corticotropin and that responsible for the hydrolysis of triglyceride in adipose tissue in the absence of this hormone.

Acknowledgment

We are grateful to Miss Mary E. Pasciuto for her assistance in this study.

REFERENCES

1. ROSENBERG, I. N.: *Proc. Soc. Exper. Biol. and Med.* **82:** 701. 1953.
2. WHITE, J. E. AND F. L. ENGEL: *J. Clin. Invest.* **37:** 1556. 1958.
3. ASTWOOD, E. B., M. S. RABEN, R. W. PAYNE AND A. B. GRADY: *J. Am. Chem. Soc.* **73:** 2969. 1951.
4. RABEN, M. S.: *Science* **125:** 883. 1957.
5. HOLLENBERG, C. H.: *Am. J. Physiol.* **197:** 667. 1959.
6. DOLE, V. P.: *J. Clin. Invest.* **35:** 150. 1956.
7. ENGEL, F. L. AND J. E. WHITE: *Am. J. Nutrition*, **8:** 691. 1960.
8. RABEN, M. S. AND C. H. HOLLENBERG: *J. Clin. Invest.* **39:** 435. 1960.

9. Bally, P. R., G. F. Cahill, Jr., B. Leboeuf and A. E. Renold: *J. Biol. Chem.* **235:** 333. 1960.
10. Gordon, R. S., Jr. and A. Cherkes: *J. Clin. Invest.* **35:** 206. 1056.
11. Gordon, R. S., Jr. and A. Cherkes: *Proc. Soc. Exper. Biol. and Med.* **97:** 150. 1958.
12. Raben, M. S. and C. H. Hollenberg: *J. Clin. Invest.* **38:** 484. 1959.
13. Knobil, E.: *Proc. Soc. Exper. Biol. and Med.* **101:** 288. 1959.
14. Raben, M. S. and C. H. Hollenberg: *Ciba Found. Coll. Endocr.* **13:** 89, London, Churchill, 1960.
15. Korn, E. D.: *J. Biol. Chem.* **215:** 1. 1955.
16. Korn, E. D. and T. W. Quigley, Jr.: *Biochim. et Biophys. Acta* **18:** 143. 1955.
17. Korn, E. D. and T. W. Quigley, Jr.: *J. Biol. Chem.* **226:** 833. 1957.
18. Cherkes, A. and R. S. Gordon, Jr.: *J. of Lipid Research* **1:** 97. 1959.
19. Páv, J. and J. Wenkeová: *Nature* **185:** 926. 1960.
20. Mashburn, L., R. Brown and W. S. Lynn: *Fed. Proc.* **19:** 224. 1960.
21. Rizack, M. A.: *Fed. Proc.* **19:** 221. 1960.

CORTISOL STIMULATION OF GLYCOGEN SYNTHESIS IN FASTED RATS[1]

JAMES ASHMORE, FRANCIS STRICKER, WILLIAM C. LOVE[2] AND GRACE KILSHEIMER

Department of Biochemistry, Indiana University Medical School, Indianapolis, Indiana

ABSTRACT

The acute effects of cortisol that result in an increase in liver glycogen deposition were studied in fasted rats, using $C^{14}O_2$ and glucose-6-C^{14} as labeled substrates. The incorporation of labeled carbon into blood glucose, liver glycogen and liver dicarboxylic acids was determined. The results of these experiments indicate that cortisol increases the incorporation of label from radioactive bicarbonate into liver glycogen without any significant increase in the activity of either blood glucose or liver dicarboxylic acids. Studies with labeled glucose showed increased deposition of glycogen from this substrate. There was little recycling of C^{14} from glucose *via* the Cori cycle. Under these conditions cortisol did not appear to increase hepatic glucose production as measured by changes in specific activity of blood glucose.

Evidence is presented that in the fasting adrenalectomized animal there is some specific impairment in glycogen formation apart from the ability of the liver to form glucose. It is suggested that the adrenal cortical steroids may act to increase glycogen deposition from blood glucose.

THAT adrenal cortical steroids promote gluconeogenesis and deposition of hepatic glycogen is well known (1, 2). These effects have been adequately documented in the classic report of Long, Katzin and Fry (3). In a number of instances it has been reported that addition *in vitro* of adrenal cortical preparations will increase glycogen deposition in liver slices (4, 5); however such effects have not been consistantly demonstrated. In the fasting rat treated with cortisol no increase in liver glycogen is observed during the first three hours following injection of the steroid (6), although significant deposition occurs from the third to sixth hours. The ability of the liver to make glucose in the fasting adrenalectomized rat appears to be somewhat limited. Lewis *et al.* (7) found that glucose formation from lactate, pyruvate or alanine was markedly impaired in such animals. More recently Winternitz *et al.* (8) have determined the extent of glycogen formation in fasting normal and adrenalectomized rats injected with a number of carbohydrate intermediates. Adrenalectomized rats formed little glycogen when glucose, fructose, glycerol, lactate or malate

Received June 30, 1960.

[1] Supported in part by PHS Grant A2701 and Eli Lilly Co.

[2] Predoctoral Fellow of the Arthritis and Rheumatism Foundation.

were administered. Cortisol restored glycogen formation in all cases. The lack of glycogen formation in the absence of the adrenal has been attributed to some defect in glycogen synthesis rather than any impairment in substrate utilization or glucose synthesis. Winternitz *et al.* (8) have suggested that a diversion of glucose-6-phosphate into an alternate route of metabolism, possibly the phosphogluconate shunt, might result in the decreased glycogen synthesis observed.

In order to better define the source of glycogen carbons following cortisol stimulation, fasting normal and adrenalectomized rats were injected with C^{14} bicarbonate and C^{14} glucose and the specific activity of liver glycogen and blood glucose followed with time after cortisol administration. Labeled bicarbonate was used since gluconeogenesis from three carbon compounds would require CO_2 fixation in the liver (9). Incorporation of C^{14} into intermediate dicarboxylic acids of the citric acid cycle was followed using a standard silica gel column technique (10). The possible contribution of the Cori cycle to glycogen formation was estimated by injecting glucose-6-C^{14}. Since reincorporation of lactic acid from muscle metabolism would result in randomization of the label into carbons 1 and 6 of glucose, activity in the top three carbons of glucose was determined by periodate oxidation of glucosephenylosazone (11). The results of these studies indicate that blood glucose is the most probable source of the liver glycogen formed by cortisol stimulation.

MATERIALS AND METHODS

Male Wistar rats 150 to 180 gm. body weight were fasted for 18 hours prior to injection of 5 mg. of cortisol (free alcohol in microcrystalline suspension injected subcutaneously) and radioactive substrates were given 3 hours after the cortisol injection. 50 μc of $C^{14}NaHCO_3$ (Sp. A. 2.5 mc/mmole) were given intraperitoneally in isotonic saline and 5 μc of glucose-6-C^{14} (Sp. A., 1 mc/mmole) were injected into the femoral vein under light ether anesthesia. Adrenalectomized animals were prepared by bilateral removal of the adrenals four days prior to the experiment and animals were maintained on isotonic saline *ad libitum*.

Blood samples for determination of the specific activity of glucose and CO_2 were obtained from the severed end of the tail. CO_2 from acidified samples of blood was permitted to diffuse into alkali and precipitated and counted as $BaCO_3$, blood glucose concentration was determined by the Somogyi-Nelson method (12) and glucosazones were prepared from the same filtrate after adding carrier glucose (13). Liver glycogen was isolated by alcohol precipitation of a KOH digest and hydrolyzed with 5 N sulfuric acid (14). Glycogen was determined as glucose by copper reduction (12) and counted as glucosephenylosazone.

The C^{14} content of the top three carbons of the glucose molecule was determined by oxidation of glucosephenylosazone with periodate (11). The mesoxaldehyde formed was recrystallized from 60% alcohol, plated and counted.

For the determination of dicarboxylic acids livers were rapidly removed, frozen in a dry ice bath, weighed and ground frozen. Proteins were removed with perchloric acid and the supernatant fluid neutralized with KOH (pH 6–7) and evaporated to dryness under reduced pressure. The dry residue was acidified with 0.5 ml. of 0.5N sulfuric acid and mixed with 0.5 gm. of silicic acid and chloroform. This was added to the top of a

silicic acid column (12.5 gm. silicic acid) and the acids eluted with butanol (10). Two ml. fractions were collected and titrated with 0.02N NaOH using phenol red indicator. Pooled fractions were evaporated under reduced pressure. An aliquot of each fraction was assayed for C^{14} by direct plating. Combined fractions were rechromatographed and counted.

RESULTS

Experiments on the incorporation of $C^{14}O_2$ into liver glycogen and blood glucose are summarized in Table 1. All animals were fasted for 21 hours prior to the experimental period, cortisol or saline were injected three hours before bicarbonate and lactate were given, and the animals were

TABLE 1. ROLE OF THE ADRENAL CORTEX IN $C^{14}O_2$ INCORPORATION INTO LIVER GLYCOGEN AND BLOOD GLUCOSE OF FASTED RATS

No. obs.	Liver glycogen		cpm/gm. liver	cpm/100 gm. body wt.	Blood glucose Sp.A., cpm/μM		
	μM/gm.	Sp.A cpm/μM			30′	60′	90′
			Fasted Normal Rats				
6	33	292	9,760	30,000	1,200	950	500
SE	0.95	55	1,800	7,400	230	200	20
			Fasted Rats Injected with Cortisol				
4	47	670	27,000	117,000	1,400	1,100	510
SE	.21	41	1,300	8,600	610	250	10
			Fasted Adrenalectomized Rats				
4	7	68	457	1,900	1,200	740	390
SE	.7	4.2	61	510	450	70	40
			Fasted Normal Rats Injected with 1 millimole Na Lactate				
6	69	520	34,000	127,000			
SE	8.2	14	3,100	15,700			
			Fasted Adrenalectomized Rats Injected with 1 millimole Na Lactate				
4	5	86	453	1,760	1,590	670	482
SE	0.6	1.4	42	450	510	90	20

killed 90 minutes after the C^{14} was injected. The glycogen content of the liver is expressed in terms of micromoles of glucose per gm. and the C^{14} content has been expressed as specific activity as well as cpm/gm. of liver and cpm/100 gm. body weight. Cortisol resulted in a small increase in the glycogen content of the liver during the experimental period in control rats, as shown many times heretofore, and both specific activity and total cpm. incorporated into liver glycogen were increased by the hormone. Although $C^{14}O_2$ incorporation into glycogen was increased about three-fold, no marked increase in the specific activity of blood glucose was observed in the cortisol treated rats.

Little glycogen was found, as anticipated, in the livers of fasting adrenalectomized rats and the incorporation of radioactivity into the glycogen fraction was less than 10% of that observed in normal animals. Blood glucose, on the other hand was labeled to about the same extent as in the normal control series.

Unlabeled sodium lactate (1 millimole) was given along with the radioactive bicarbonate in a second series of experiments. The effect of lactate

to increase CO_2 fixation is well known (15), and the results achieved in normal animals are consistent with the experience of others. Since both cortisol and lactate increase the incorporation of radioactivity from $C^{14}O_2$ into liver glycogen, the action of the steroid may be only to increase the availability of three carbon compounds to the liver. The failure of lactate to stimulate incorporation of C^{14} into liver glycogen in the fasting adrenalectomized rats suggests some defect in glycogen formation.

One of the several possible actions of cortisol is that of accelerating glucose metabolism *via* the Cori cycle. Thus, glucose removed by extrahepatic tissues could be rapidly glycolyzed and the lactic acid formed returned to the liver increasing CO_2 fixation and possibly glycogen deposition. Such an effect would be consistent with the postulate of Henneman and Bunker (16) that corticoids have a direct inhibitory action on the

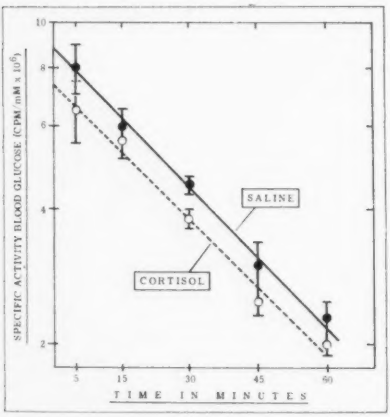

FIG. 1. Log specific activity of blood glucose has been plotted as a functoin of time after intravenous injection of 5 μc of glucose-6-C^{14} into cortisol treated and saline control rats. Each curve represents the mean of three experiments and total spread of points is indicated (1).

oxidation of pyruvate. In order to test this hypothesis animals were injected with glucose-6-C^{14}. The semi-logarithmic decay curves for blood glucose specific activity in control and cortisol treated rats are given in Figure 1. An increase in hepatic glucose production would be expected to result in a more rapid decrease in blood glucose specific activity in the cortisol treated animals. This was not observed. An increase in the Cori cycle under these conditions would result in randomization of the label of the glucose molecule in carbon atoms 1 and 6. The extent of randomization was determined by periodate oxidation of the phenylglucosazone and was found to be neligible, amounting to about 5% of the circulating radioactivity at the end of one hour. No difference was found between control and cortisol treated animals.

Incorporation of carbon from glucose-6-C^{14} into tissue glycogen of fasted normal and cortisol treated rats is given in Table 2. C^{14} incorpora-

TABLE 2. INCORPORATION OF CARBON FROM GLUCOSE-6-C^{14} INTO GLYCOGEN OF FASTED NORMAL AND CORTISOL TREATED RATS

	μM/gm.	Glycogen, Sp.A. cpm/μM	cpm/gm. wet tissue
		Liver	
Control	6.8	212	1,440
	6.1	182	1,110
	6.3	198	1,250
(Mean)	6.4	197	1,275
Cortisol	22.	242	5,300
	35	147	5,150
	26	192	5,000
(Mean)	28	194	5,150
		Muscle	
Control	21	6.6	138
	13	6.9	90
	12	8.8	105
(Mean)	15	7.1	111
Cortisol	15	10.4	160
	22	9.2	202
	23	13.2	313
(Mean)	20	10.9	225

tion into both liver and muscle glycogen was increased by cortisol. The extent of glycogen labeling reflects the amount of glycogen found in the liver and suggests that in both cases most of the glycogen isolated has been formed from blood glucose of the same specific activity. The increase in radioactivity of muscle glycogen in cortisol treated animals indicates an increase in glycogen deposition. This may be due in part to the slightly higher blood glucose values in the cortisol treated series.

We have observed in experiments with radioactive bicarbonate that incorporation of C^{14} into liver glycogen continues for some time after most of the radioactive CO_2 has disappeared from the blood (17). These experiments suggested that during the first hour after $C^{14}O_2$ administration, a pool of labeled intermediates might be formed from which labeled glycogen could be made over the next few hours. Since initial fixation of CO_2 would result in labeling of the dicarboxylic acids of the citric acid cycle, these compounds were isolated from the liver one hour after radioactive bicarbonate or glucose were given. The results of one experiment are given in Figure 2. Cortisol acting over a period of 4 hours in the fasted rat did not cause any pronounced increase in any of the components isolated. Mean values for the C^{14} activity found in these fractions are given in Table 3. Although an increase in the activity of the lactate-succinate fraction from $C^{14}O_2$ was observed in the cortisol series, the amount of activity found is not sufficient to account for the glycogen formed during the following hour.

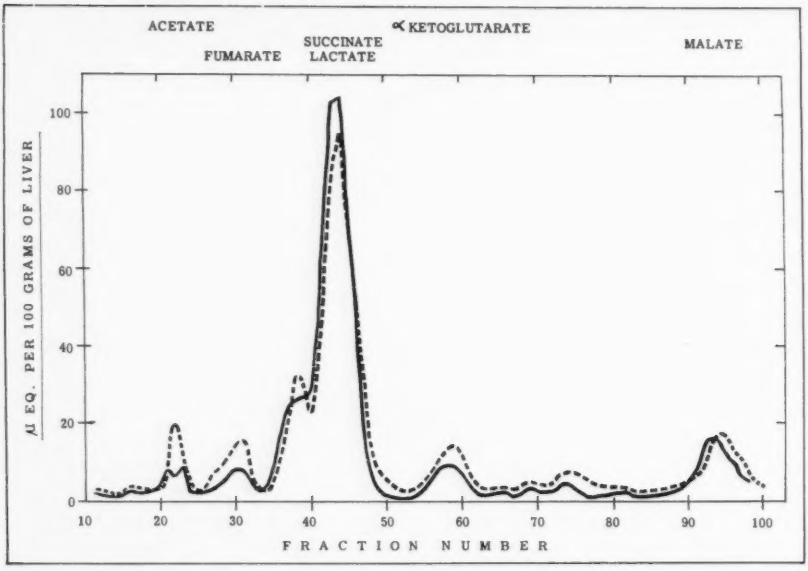

FIG. 2. Microequivalents of acid per 100 gm. of wet liver has been plotted as a function of fraction number. The volume of each fraction was 2 ml. and a gradient elution of butanol in chloroform was used.

DISCUSSION

These experiments deal primarily with the acute effect of cortisol to increase liver glycogen deposition and should not be confused with later effects of the steroid that result in increased gluconeogenesis. Previous experiments by Winternitz *et al.* (8) have demonstrated that in the fasting adrenalectomized rat there is presumably some specific impairment in glycogen formation. Our results obtained in adrenalectomized rats are in agreement with this conclusion. Although radioactive carbon from labeled bicarbonate was found in blood glucose to about the same extent as in control animals, incorporation of label into glycogen was markedly diminished. Such a defect probably involves some biochemical transformation between glucose-6-phosphate and glycogen.

TABLE 3. INCORPORATION OF C^{14} INTO LIVER CARBOXYLIC ACIDS
(Each value represents the mean of pooled samples from three rats. Values are expressed as cpm/gm. liver)

Acid fraction	Glucose-6-C^{14}		$C^{14}O_2$	
	Control	Cortisol	Control	Cortisol
Fumarate	200	220	20	70
Lactate-Succinate	6,100	6,300	250	580
Ketoglutarate	550	500		
Malate	430	670	230	260

In cortisol treated normal animals it has been possible to show an increase in liver glycogen deposition in the absence of any pronounced increase in gluconeogenesis. That is, all animals appear to incorporate label from radiobicarbonate into blood glucose at essentially the same rate. The turnover rate of blood glucose was not increased, nor was there any evidence of an increase in recycling of the label. It was also found that cortisol under these conditions did not increase liver dicarboxylic acids and further the incorporation of labeled carbon into these fractions was not stimulated. Therefore, it would appear unlikely that the initial glycogen deposition observed resulted from either an increase in substrates to the liver or an increase in CO_2 fixation.

The most probable source of the glycogen laid down under initial cortisol stimulation is the blood glucose. Experiments with labeled glucose indicate that cortisol does stimulate incorporation of label from this substrate into glycogen. Specific activity curves of blood glucose and liver glycogen (17) obtained with radioactive bicarbonate are consistent with the classical precursor curves of Zilversmit *et al.* (18) and support our contention that glucose may serve as the precursor. Winternitz *et al.* (8) have suggested the possibility that the corticoids may control some alternate pathway of glucose-6-phosphate metabolism, perhaps the phosphogluconate shunt. Such an effect could result in increased glycogen formation without much apparent change in the rate of blood glucose formation. However, estimations of the activity of the phosphogluconate shunt in liver slices have not revealed any pronounced effect of adrenal hormones on this metabolic route (19).

Some caution should be exercised in accepting these tentative conclusions. It is possible that slight changes in the rates of a number of reactions may result, through a combination of effects, in a marked increase in glycogen deposition without much significant change in any of the other parameters measured.

REFERENCES

1. Russell, J.A.: Ciba Foundation Colloquia on Endocrinology (G. E. W. Wolstenholme, Ed.), Internal Secretions of the Pancreas, Vol. VI, p. 193. Little, Brown and Company, Boston, 1953.
2. Long, C. N. H.: Ciba Foundation Colloquia on Endocrinology (G. E. W. Wolstenholme, Ed.), Internal Secretions of the Pancreas, Vol. VI, p. 136. Little, Brown and Company, Boston, 1953.
3. Long, C. N. H., B. Katzin and E. G. Fry: *Endocrinology* **26:** 309. 1940.
4. Chiu, C. Y. and D. M. Needham: *Biochem. J.* **46:** 114. 1950.
5. Chiu, C. Y.: *Biochem. J.* **46:** 120. 1950.
6. Hyde, P. M.: *Endocrinology* **61:** 774. 1957.
7. Lewis, R. A., D. Kuhlman, C. Delbue, G. F. Koepf and G. W. Thorn: *Endocrinology* **27:** 971. 1940.
8. Winternitz, W. W., R. Dintzis and C. N. Long: *Endocrinology* **61:** 724. 1957.
9. Krebs, H. A.: *Bull. Johns. Hopkins Hospital* **95:** 19. 1954.

10. Donaldson, K. O., V. J. Tulane and L. M. Marshall: *Anal. Chem.* **24:** 182. 1952.
11. Landau, B. R., A. B. Hastings and F. B. Nesbett: *J. Biol. Chem.* **214:** 525. 1955.
12. Nelson, N.: *J. Biol. Chem.* **153:** 375. 1944.
13. Ashmore, J., G. F. Cahill, Jr., A. S. Earle and S. Zottu: *Diabetes* **7:** 1958.
14. Good, C. A., H. Kramer and M. Somogyi: *J. Biol. Chem.* **100:** 485. 1933.
15. Buchanan, J. M. and A. B. Hastings: *Physiol. Rev.* **26:** 120. 1946.
16. Henneman, D. H. and J. P. Bunker: *Am. J. Med.* **23:** 34. 1957.
17. Ashmore, J.: *Rev. Canad. Biol.* **18:** 229. 1959.
18. Zilversmit, D. B., C. Entenman and M. C. Fishler: *J. Gen. Physiol.* **26:** 325. 1943.
19. Ashmore, J., G. F. Cahill, Jr., R. Hillman and A. E. Renold: *Endocrinology* **62:** 621. 1958.

THE THYROID AND ABSORPTION OF VITAMIN B_{12} IN RATS[1]

KUNIO OKUDA AND BACON F. CHOW

Department of Biochemistry, School of Hygiene & Public Health, The Johns Hopkins University, Baltimore, Maryland

ABSTRACT

The absorption of vitamin B_{12} and the concentrations of vitamin B_{12} in the plasma and liver were measured in normal rats and in rats in which the thyroid activity was altered. It was found that the absorption of an oral dose of radioactive vitamin B_{12} is markedly reduced after thyroidectomy and that the thyroid hormone treatment readily normalizes absorption in thyroidectomized rats. However, the administration of intrinsic factor (IF) prepared from rat stomachs did not improve absorption in such animals. The measurement of IF activity in the stomach demonstrated that the amount of IF in the stomach is not reduced in thyroidectomized rats. It seems, therefore, that the thyroidal influence on the absorption of vitamin B_{12} is not mediated through the production of IF. A possible hormonal regulation acting directly on the intestinal wall for the absorption of vitamin B_{12} has been discussed.

STUDIES on the mechanism of absorption of vitamin B_{12} have centered primarily on the role of intrinsic factor (IF), little on the regulatory mechanism for intestinal absorption of this vitamin under various abnormal conditions. In our previous report, it was demonstrated that levels of vitamin B_{12} in plasma of human cretins are low and can be elevated after thyroid therapy (1). These data suggest the possibility that thyroid activity has a regulatory effect on the absorption of vitamin B_{12}. We, therefore, studied the influence of thyroid activity on the absorption of vitamin B_{12} and its concentration in tissues and plasma of animals which were made athyroid or fed thyroid hormone in the diet. This communication reports findings of such studies which demonstrated a hormonal regulation of intestinal absorption of vitamin B_{12}. Efforts were also made to elucidate the control mechanism, particularly with reference to the effect of thyroid activity on the production intrinsic factor which is known to aid absorption of vitamin B_{12}.

MATERIALS AND METHODS

Preparation of animals. (a) Thyroidectomized rats. In studies in which absorption of radioactive vitamin B_{12} was measured by fecal excretion test, thyroidectomized and control rats were obtained from one source.[2] In other studies, these two groups of rats

Received July 5, 1960.

[1] The authors wish to acknowledge with thanks a grant-in-aid from USPHS RG-4916.

[2] Kindly supplied by Armour Research Laboratories.

were purchased from a commercial source.[3] (b) Rats fed with desiccated thyroid. Young adult rats of the McCollum strain from our own colony were fed desiccated thyroid in the basal diet at the level of 0.1 to 0.2% for a period of more than three weeks. Loss of weight in comparison with the control animals was taken as the indication of a hyperthyroid state. The experimental basal diet consisted of 20% vitamin-free casein, 5% mazola oil, 71% sucrose, 4% salt mixture by weight, with all the vitamins except vitamin B_{12}. (c) Thiouracil treated rats. In a study of the effect of chronic hypothyroidism on plasma vitamin B_{12} levels, rats from our stock colony were fed vitamin B_{12} free soybean diet and were given *ad lib*, drinking water containing 0.05% thiouracill

Measurement of vitamin B_{12} in plasma and liver. Plasma and liver specimens were processed according to the procedures previously described (2, 3). They were assayed for the microbiological activity of vitamin B_{12} with Skeggs' medium (4) and *Lactobacillus leichmannii* 4797 as the test organism.

Measurement of absorption of vitamin B_{12}. A single dose of 50 mμg of radioactive vitamin B_{12}[4] was administered orally to fasting rats. The radioactivity in the fecal specimen collected for 5 days and in certain organs was taken as an index of absorption. The technique of radioactivity measurement of such specimens with a well-type gamma scintillation counter has been described (5).

Preparation of intrinsic factor from the stomach of untreated rats. The fundus mucosa of the stomach of normal rats from our colony was scraped and homogenized with a physiological saline solution. After 20 minutes of centrifugation at 3,000 rpm, the supernatant fluid was used as the source of IF and its activity was tested with the "intestinal loop" technique (6). The main portion of the IF activity remains in the supernatant solution (6). This mucosal extract was used in the experiments in which IF was mixed with a test dose of radioactive vitamin B_{12} and administered to thyroidectomized rats.

Determination of total IF activity in the stomach of thyroidectomized rats. For the determination of the total content of IF, the entire stomach of experimental rats was removed and homogenized *in toto* with a physiological saline solution in the cold. The homogenate was centrifuged. The supernatant fluid was used for the measurement of vitamin B_{12} binding capacity by the dialysis method (5); and for the estimation of IF activity, by the "loop" technique (6). The latter measurement was performed by introducing into a loop prepared in a normal rat an amount of the supernatant fluid containing enough IF so as to bind with 10 mμg radioactive vitamin B_{12}. In this way, the mixture contains IF in an amount less than that needed for a maximum absorption of this dose of vitamin B_{12}. The decrease of radioactivity in the loop after 24 hours was taken as the amount absorbed. The enhancement of absorption by the addition of stomach extract prepared from the thyroidectomized rats was compared with that by the extract prepared from the normal control rats.

RESULTS

Effect of feeding desiccated thyroid on vitamin B_{12} absorption. In Study I, adult female rats were divided into two groups: Group A (6 rats) were fed the basal casein diet and Group B (7 rats) were offered the same diet supplemented with desiccated thyroid (2 gm./kg. diet) for 4 weeks. Both groups of animals were then tested for their ability to absorb vitamin B_{12}.

[3] Charles River Breeding Co.

[4] Cobalt 60 labeled vitamin B_{12} with specific activity of 1 mc/mg., supplied by Merck and Co.

The results (Table 1) demonstrate an increase in absorption according to the fecal excretion test and the uptake of radioactivity by the liver ($P < 0.05$). In Study II, four groups of young female rats were used. The first two were given the same diet as Groups A and B in Study I, which contained no added vitamin B_{12}. To study the effect of thyroid feeding on absorption in the presence of exogenous vitamin B_{12}, two additional groups of rats were used. Both groups received a diet to which vitamin B_{12} was added (250 μg/kg. diet). However Group D received desiccated thyroid supplementation. The exogenous vitamin B_{12} was removed from the diets for Groups C and D during the two days prior to the test in order to avoid isotope dilution effect by dietary vitamin B_{12}. The results of Study II

TABLE 1. ABSORPTION OF RADIOACTIVE VITAMIN B_{12} BY HYPERTHYROID RATS

Study No. (sex)	Group	No. of rats	Av. body wt. (gm.)		Absorption by fecal excretion test (mμg)	Organ uptake	
			Initial	After 4 wks.		Liver	Kidneys
						(mμg)	
I (F)	A. Control	6	176	202	18.7 ± 0.9	2.38 ± 0.09	1.90 ± 0.26
	B. Thyroid	7	185	163	29.2 ± 1.2	4.48 ± 0.37	2.22 ± 0.14
II (F)	A. Control	5	178	196	18.3 ± 1.9	3.39 ± 0.36	3.24 ± 0.36
	B. Thyroid*	3	187	145	26.0 ± 1.0	6.98 ± 0.20	4.66 ± 0.25
	C. B_{12}†	5	179	196	16.6 ± 2.0	1.76 ± 0.30	7.80 ± 0.98
	D. Thyroid* + B_{12}†	3	187	157	23.6 ± 2.6	3.14 ± 0.10	9.89 ± 1.26
III (M)	A. Control	6	344	398	24.2 ± 1.9	3.83 ± 0.30	2.67 ± 0.17
	B. Thyroid*	6	350	319	23.9 ± 0.8	4.52 ± 0.30	3.17 ± 0.17
IV (M)	A. Control	6	298	345	24.8 ± 1.7	2.28 ± 0.24	2.28 ± 0.16
	B. Thyroid*	6	289	276	23.7 ± 1.3	3.04 ± 0.25	2.10 ± 0.12

Figures are mean ± S.E.D.
A single oral dose of 50 mμg. radioactive vitamin B_{12} was given.
Animals were fed B_{12} free basal diet supplemented with the following for 4 weeks:
* 0.2% desiccated thyroid.
† 250 mμg. vitamin B_{12}/kg. diet.

likewise showed that thyroid treatment increased the absorption of vitamin B_{12}. A similar difference was observed in the organ uptake of radioactivity. It is to be noted that the kidneys of vitamin B_{12} treated animals (C and D) retained considerably more radioactivity than did those of the rats (A and B) receiving vitamin B_{12}.

Another study was conducted with adult male rats, which were fed diets containing no added vitamin B_{12}. No increase in absorption was demonstrated in this study (Study III). This study was repeated with another set of rats with similar results. Whether the difference in results between experiments with females and males may be attributed to sex or other factors such as weight differences remains to be elucidated.

Absorption of vitamin B_{12} by thyroidectomized rats and effect of feeding thyroid hormone. To determine the effect of thyroidectomy and of feeding thyroid

hormone on vitamin B_{12} absorption, by these operated rats, one-half of a group of two-month old male animals were thyroidectomized, and the other half served as control. Both groups of rats were fed the stock diet for one month and given the B_{12} absorption test. The data of such study (Table 2, Study A) show that the absorption of radioactive vitamin B_{12} by the thyroidectomized rats was markedly lower than that of the control, being 6.2 ± 1.1 mμg and 25.5 ± 1.0 mμg, respectively. The thyroidectomized rats were then given desiccated thyroid in the stock diet (0.1%) for 3 weeks, whereas the control rats remained on the stock diet. They were again given the absorption test. The absorption by the thyroidectomized rats after the thyroid powder treatment was found to be markedly increased, from the previous average of 6.2 to 27.7 mμg. The latter value was slightly but insignificantly higher than the absorption of the control group. A similar study (B) was conducted in which l-thyroxine (2.5 μg/10 gm. diet) was fed to the thyroidectomized rats in place of desiccated thyroid. The data again demonstrated the marked diminution of absorption by thyroidectomized rats which could be corrected by thyroxine feeding (compare 12.7 ± 2.5 mμg and 23.0 ± 2.0 mμg). These two groups of thyroidectomized rats were obtained at different times, and the difference in the aborption of vitamin B_{12} before the thyroid hormone treatment may be due to some variation associated with the batch of the animals.

TABLE 2. ABSORPTION OF VITAMIN B_{12} BY THYROIDECTOMIZED RATS AND ENHANCEMENT OF ADSORPTION BY THYROID HORMONE TREATMENTS

Study	Group	No. rats	Body wt. (gm.)	B_{12} absorption by fecal excretion test†	
				Before treatment	After thyroid treatment
A	Control	5	304	25.5 ± 1.0	23.6 ± 2.3
	Thyroidectomized*	5	299	6.2 ± 1.1	27.7 ± 1.8
B	Control	3	370	27.4 ± 0.8	28.7 ± 1.0
	Thyroidectomized**	5	265	12.7 ± 2.5	23.0 ± 2.0

* Desiccated thyroid, 0.1% in diet, was given to thyroidectomized rats in Study A: L-thyroxine, 2.5 μg per day in diet, was given to thyroidectomized rats in Study B.
† All results are expressed as mμg ± S.E.D.

Plasma and liver concentrations of vitamin B_{12} in rats with varying thyroid activities. If thyroid activity does influence vitamin B_{12} absorption, this regulatory process may reflect itself in the vitamin B_{12} levels of plasma or liver of the animals according to the state of thyroid activity. For this reason, vitamin B_{12} concentrations in plasma and liver of the rats whose thyroid activity was altered were determined. In the first study, the four groups of animals used in the previous absorption experiment (Table 1, Study II) were sacrificed immediately after the test and vitamin B_{12} concentrations in the plasma and liver homogenate were determined. The

TABLE 3. EFFECT OF THE ADMINISTRATION OF DESICCATED THYROID ON VITAMIN B_{12} LEVELS IN THE PLASMA AND LIVER

Group	No. of rats	Plasma B_{12} level at 4 wks. (mμg/cc.)	Liver B_{12} concentration at 5 wks. (mμg/gm.)
A. Control	5	0.75 ± 0.09*	41 ± 5.0*
B. Desiccated Thyroid	3	0.83 ± 0.02	36 ± 0.2
C. B_{12}	5	1.21 ± 0.06	196 ± 15.5
D. Desiccated Thyroid & B_{12}	4	1.31 ± 0.07	282 ± 33.0

Plasma vitamin B_{12} was determined by titrimetric method.
* Standard error of the mean.

results (Table 3) show that desiccated thyroid supplementation increased the liver vitamin B_{12} levels when exogenous B_{12} was present, but this difference was not observed in the absence of added vitamin B_{12}. Only slight differences were observed in the plasma B_{12} levels.

In another study, 2-month old male rats raised on our stock diet containing liver powder were used. One-third of these animals were thyroidectomized, another third were placed on the same diet containing desiccated thyroid (0.2%), and another third received no treatment. One month afterwards, vitamin B_{12} concentrations in the plasma and liver were determined. As shown in Table 4, hyperthyroid rats had a higher liver B_{12} level than those in the control group, while the thyroidectomized rats had the highest. However, the thyroidectomized rats had considerably lower plasma levels of vitamin B_{12} than those in other groups. Another study was conducted with two groups of rats (one, thyroidectomized; and the other, control). Vitamin B_{12} levels were measured two months after thyroidectomy. Again, the thyroidectomized rats showed lower plasma levels and higher liver concentrations of vitamin B_{12}.

Since these experiments indicated that athyroid rats absorb only one-fourth to one-half of the test dose of vitamin B_{12}, and that plasma level of vitamin B_{12} decreases after the operation, it seemed that suppression of the thyroid function with a chemical agent would also bring about a similar effect. To this end, three month old female rats, which had been

TABLE 4. THE PLASMA AND LIVER CONCENTRATIONS OF VITAMIN B_{12} IN HYPER- AND ATHYROID RATS

Study	Group	No. of rats	Body wt. at sacrifice (gm.)	Plasma B_{12} (mμg/cc.)	Liver B_{12} Concentration (mμg/cc.)
A (1 mo.)*	Control	5	335	1.03 ± 0.04‡	71.4 ± 3.3‡
	Hyperthyroid†	3	235	1.08 ± 0.03	93.0 ± 2.2
	Thyroidectomized	5	255	0.86 ± 0.05	153.0 ± 40.0
B *q* (2 mo.)*	Control	4	363	0.98 ± 0.06	63.8 ± 6.6
	Thyroidectomized	4	158	0.75 ± 0.07	117.0 ± 18.1

* Months after thyroidectomy.
† Fed 0.2% desiccated thyroid for 1 month.
‡ Standard error of the mean.

TABLE 5. EFFECT OF THIOURACIL ADMINISTRATION ON PLASMA LEVELS OF VITAMIN B_{12}

Group	No. of rats	Initial	Body wt. (gm.) 5 months	Plasma B_{12} (mμg/cc.)
Control	16	210	229	0.22 ± 0.020‡
Thiouracil*	7	189	200	0.23 ± 0.020
B_{12}†	12	219	225	1.23 ± 0.035
B_{12} & Thiouracil	7	202	216	0.67 ± 0.100

* 0.05% in drinking water.
† 50 mμg/kg. soybean diet.
‡ Standard error of the mean.

fed the stock diet, were divided into four groups: two groups were fed a vitamin B_{12} deficient soybean diet, and the other two groups were fed the same diet supplemented with vitamin B_{12} (50 μg/kg. diet). One each of the two groups received thiouracil in drinking water (0.05% solution). After 5 months of the feeding, blood was drawn by heart puncture and plasma vitamin B_{12} was determined. The results (Table 5) showed that the plasma levels of vitamin B_{12} of the thiouracil treated rats fed on the vitamin B_{12}-soybean diet were significantly lower ($P<0.001$) than those of the rats receiving vitamin B_{12} but no thiouracil, the average being 0.67 ± 0.100 and 1.23 ± 0.035 mμg/ml., respectively. There was no demonstrable difference in plasma vitamin B_{12} between the two groups which received no vitamin B_{12} in the diet.

Effect of IF on vitamin B_{12} absorption by thyroidectomized rats. In order to ascertain whether reduction in absorption after thyroidectomy is associated with a possible defect in IF production, experiments were carried out in which a saline extract of normal rat stomach was administered concomitantly with a test dose of radioactive vitamin B_{12} to thyroidectomized rats. The results of three such studies showed essentially that the addition of IF in the form of stomach extract did not improve the absorption, as shown in Table 6 in which the results of a typical experiment are given.

It is possible that IF administered concomitantly with the test dose by mouth may be inactivated by the gastric juice before it can exert its action

TABLE 6. EFFECT OF RAT STOMACH EXTRACT ON THE ABSORPTION OF VITAMIN B_{12} IN THYROIDECTOMIZED RATS

Group	No. of rats	Absorption by fecal test (mμg)	Organ uptake of radioactivity (mμg)	
			Liver	Kidneys
Control	4	21.0 ± 2.6†	1.45 ± 0.31†	2.56 ± 0.41†
Thyroidectomized	3	8.6 ± 3.1	0.47 ± 0.11	0.64 ± 0.20
Thyroidectomized with stomach extract*	3	7.4 ± 2.1	0.45 ± 0.09	0.62 ± 0.21

* Contained vitamin B_{12} binding capacity of 200 mμg.
† Standard error of the mean.

in the intestine in rats, even though this is not true in man. Therefore, the following experiment was carried out: both control and thyroidectomized rats were laparotomized. While fasting, a mixture of radioactive vitamin B_{12} and rat stomach extract containing 100 mμg B_{12} binding power was directly injected into the duodenum, and the absorption was measured. The results again showed (Table 7) that the addition of IF in the form of stomach extract did not improve the absorption, in the thyroidectomized rat (Table 6) while apparently doing so to a certain extent in the controls.

TABLE 7. ABSORPTION OF RADIOACTIVE VITAMIN B_{12} DIRECTLY INFUSED INTO THE DUODENUM AND THE EFFECT OF STOMACH EXTRACT ON ABSORPTION

Group	No. of rats	Absorption by fecal test (mμg)	Organ uptake of radioactivity (mμg)	
			Liver	Kidneys
Control (without extract)*	3	8.0 ± 1.1‡	1.30 ± 0.17‡	2.48 ± 0.24‡
Control (with extract)†	5	17.9 ± 2.6	2.14 ± 0.38	4.41 ± 0.78
Thyroidectomized (with extract)†	4	6.6 ± 2.3	1.00 ± 0.26	1.06 ± 0.41

* 50 mμg Co60-B_{12} alone.
† 50 mμg Co60-B_{12} mixed with stomach extract containing 100 mμg B_{12} binding power.
‡ Standard error of the mean.

Measurement of the amount of IF present in the stomach. It has been found in this laboratory (6) that IF activity of the stomach mucosa extract measured by the "loop" technique is proportional to its vitamin B_{12} binding capacity. Therefore, the total IF activity present in the stomach could be estimated from the total binding power of the whole stomach. Hence, the whole stomach of the control and the thyroidectomized rat was homogenized and the vitamin B_{12} binding capacity was measured and compared. The results demonstrated no difference in the total vitamin B_{12} binding power of the stomach of thyroidectomized rats and normal rats, which were 1310 ± 151 mμg and 1336 ± 91 mμg, respectively.

It has also been shown by the "loop" method that the maximum absorption of 10 mμg of test dose of vitamin B_{12} takes place in the presence of that amount of extract capable of binding about 15 mμg B_{12}. In order to determine whether or not there is the same correlation between vitamin B_{12} binding power and IF activity in the thyroidectomized rats, stomach extract which was used in the preceding experiment was further tested for IF activity. An aliquot containing a B_{12} binding power of 10 mμg was mixed with 10 mμg test dose of Co60-B_{12} and applied into 3 loops prepared in 3 normal rats, and the absorption was measured. It was found that the IF activity of the extract prepared from the thyroidectomized rat was practically the same as that of the control rat (Table 8). It seems,

TABLE 8. IF ACTIVITY OF STOMACH EXTRACT OF THE THYROIDECTOMIZED AND CONTROL RATE AS MEASURED BY "LOOP" TECHNIQUE

Source of stomach extract	No. of loops used	Absorption from loop (mμg)	Organ uptake of radioactivity (mμg)	
			Liver	Kidneys
B_{12} alone	4	1.1 ± 0.01‡	0.15 ± 0.03‡	0.32 ± 0.03‡
Control rat #1*	3	5.3 ± 0.12*	0.67 ± 0.12	1.78 ± 0.06
Thyroidectomized rat #2†	3	5.3 ± 0.25*	0.73 ± 0.07	1.79 ± 0.06

* Had total binding of 1460 mμg per stomach.
† Had total binding of 1468 mμg.
‡ Standard error of the mean.
An amount of extract containing 10 mμg B_{12} binding power was used with the test dose of 10 mμg of Co60-B_{12}.

therefore, that the ratio of the B_{12} binding power to IF activity is the same both in normal and thyroidectomized rats and that the total amount of IF present in the stomach mucosa is not reduced in the latter.

DISCUSSION

Our data presented in this report show that thyroidectomy reduces the absorption of vitamin B_{12} with a concomitant decrease in the concentration of vitamin B_{12} in plasma. The reduction of thyroid activity by thiouracil feeding for a long period produced a similar effect on the plasma vitamin B_{12}. However, under our experimental conditions, the hepatic level of vitamin B_{12} in thyroidectomized rats was not lowered, thus creating an abnormal ratio of B_{12} concentration in the plasma to that in the liver. This phenomenon may indicate an impaired metabolism of this vitamin. The administration of desiccated thyroid or thyroxine to such animals readily restored the absorption to normal, thus demonstrating a regulatory effect of the thyroid on vitamin B_{12} absorption.

The effect of the administration of thyroid hormone to normal rats on the absorption of vitamin B_{12} varied with sex. Although females which were given dietary thyroid powder absorbed more of the test dose of vitamin B_{12} than did the controls, this was not true of the male rats. Whether the effect of thyroid feeding varies with the sex of rats, or sex hormones was interrelated in the regulatory mechanism of absorption by the thyroid hormone, or even due to differences of body weights, is not clear at the moment.

The absorption of vitamin B_{12} requires IF and probably some other factors. Therefore, attempts were made to determine whether the reduction in absorption in athyroidism is due to reduction in IF production from the stomach. Such studies demonstrated that addition of IF to the test dose of vitamin B_{12} did not improve absorption at all in thyroidectomized rats. Furthermore, when the total IF activities in the stomach of the thyroidectomized and control rats were estimated, no difference was found. That the secretion of IF into the gastric juice is reduced in thy-

roidectomized rats seems to be unlikely, because the experiment in which an excess amount of IF was mixed with the test dose of vitamin B_{12} and introduced directly into the duodenum of both thyroidectomized and normal rats showed that the absorption in the former was still poorer than that in the latter. These findings, therefore, suggest that the regulatory effect of the thyroid hormone on vitamin B_{12} absorption is not mediated through the production of IF, but through some sytem which is probably present in the intestinal wall.

REFERENCES

1. Hellegers, A., K. Okuda, R. E. L. Nesbitt, Jr., D. W. Smith and B. F. Chow: *Am. J. Clin. Nutr.* **5**: 327. 1957.
2. Okuda, K., R. D. Wood, C. A. Lang and B. F. Chow: *Fed. Proc.* **13**: 464. 1954.
3. Yamamoto, R. S., K. Okuda and B. F. Chow: *Proc. Soc. Exper. Biol. & Med.* **94**: 497. 1957.
4. Skeggs, H. R., H. M. Nepple, K. A. Valentik, J. W. Huff and L. Wright: *J. Biol. Chem.* **184**: 211. 1950.
5. Okuda, K., J. A. Wider and B. F. Chow: *J. Lab. & Clin. Med.* **54**: 535. 1959.
6. Okuda, K.: *Am. J. Physiol.* **199**: 84. 1960.

BIOLOGICAL ACTIVITY OF 9α,11β-DIHALO STEROIDS[1,2]

RALPH I. DORFMAN, FRED A. KINCL
AND HOWARD J. RINGOLD

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts
and
Syntex Research Laboratories, Mexico, D.F.

ABSTRACT

Three 9α,11β-dihalo corticoids, 9α,11β-dichloro-17α,21-dihydroxy-$\Delta^{1,4}$-pregnadiene-3,20-dione-21-acetate, 9α,11β-dichloro-17α,21-dihydroxy-Δ^{4}-pregnene-3,20-dione-21-acetate, and 9α-bromo-11β-fluoro-17α,21-dihydroxy-Δ^{4}-pregnene-3,20-dione-21-acetate were studied by thymolytic, anti-inflammatory, and liver glycogen methods in the adrenalectomized rat. In all instances the substitution of the 11β-halo group for the 11β-hydroxy resulted in corticoids with significantly reduced activity.

ROBINSON *et al.* (1) have reported that 9α-bromo-11β-chloro-17α, 21-hydroxy-$\Delta^{1,4}$-pregnadien-3,20-dione-21-acetate is about 1.2 times as active as prednisolone acetate while the 9α,11β-dichloro-21-acetate and 9α,11β-dichloro compounds are 4 and 8 times as active as the same standard using a granuloma pouch assay (2). However, the latter two compounds were judged to have "only slight activity" in a dog (intact or adrenalectomized) eosinopenic assay. These data imply a rather wide separation of anti-inflammatory and carbohydrate activity for these unique corticoids. We have restudied this possibility using the following 9α,11β-dihalo steroids: 9α,11β-dichloro-17α,21-dihydroxy-Δ^{4}-pregnene-3,20-dione-21-acetate (DDΔ^{4}), 9α,11β-dichloro-17α,21-dihydroxy-$\Delta^{1,4}$-pregnadiene-3,20-dione-21-acetate (DD$\Delta^{1,4}$A), and 9α-bromo-11β-fluoro-17α,21-dihydroxy-Δ^{4}-pregnene-3,20-dione-21-acetate (BFΔ^{4}A). Various corticoid tests have been used such as rat thymus involution, anti-inflammation, and deposition of glycogen in the liver, all in adrenalectomized rats.

METHOD

Rat Thymolytic-Anti-Inflammatory Test

Twenty-three to twenty-six day old albino rats were adrenalectomized and two 5 ± 1 mg. cotton pellets inserted subcutaneously. The standard and test compounds were administered by gavage or subcutaneous injection once daily for six days. The daily dose was contained in 0.5 ml. of the aqueous suspending medium containing the test compound. The suspending medium consists of an aqueous solution of sodium chloride

Received July 5, 1960.

[1] Supported in part by Grant No. CY-2193, U. S. Public Health Service.

[2] This is Steroids CLVIII in the series published from the Syntex Research Laboratories.

(0.9%), polysorbate 80 (0.4%), carboxymethylcellulose (0.5%), and benzyl alcohol (0.9%). The control group received only the vehicle.

Twenty-four hours after the last treatment the thymus glands and granulomas formed around the cotton pellets were removed. The thymus glands were weighed after blotting on slightly moistened filter paper and the granulomas weighed after drying for 24 hours at 100° C. The net weight of the two dried granulomas was considered as a single determination for each animal. The thymus ratio was calculated as the mg. of tissue weight per gram of final body weight.

Injection Anti-Inflammatory Test

This test was performed by Dr. Elva G. Shipley of the Endocrine Assay Laboratory, Madison, Wisconsin.

Fifty-day old male rats, 10 gm. weight range, were bilaterally adrenalectomized under ether anesthesia. At the time of adrenalectomy, 4 dental cotton pellets, ranging between 6.0–8.0 mg., were subcutaneously implanted, 2 in the neck region and 2 in the pectoral region. The animals were then weighed and placed in cages where they received *ad libitum* a stock diet and 1% saline drinking water.

The test materials were injected subcutaneously once daily for 5 days, commencing on the operative day. Autopsy was on the day following the last day of treatment. At the time of autopsy, the animals were weighed and the granuloma pellets were carefully dissected out and weighed. The granulomas were then placed in an oven to dry, approximately 48 hours being allowed for complete drying, after which they were reweighed. An average dry weight was found for each animal and net weights were obtained by subtraction of the cotton pellet weight.

Glycogen Deposition Test

This test was performed by Dr. Elva G. Shipley of the Endocrine Assay Laboratories, Madison, Wisconsin, using a method employing 21-day old adrenalectomized rats previously described (3).

RESULTS AND CONCLUSION

Table 1 lists the results of DD$\Delta^{1,4}$A in the rat thymolytic and anti-inflammatory assay when the compounds were administered by gavage. In this assay the dihalo steroid was approximately equal to cortisol acetate as an anti-inflammatory agent but less than one-half as active on the

TABLE 1. THE THYMOLYTIC AND ANTI-INFLAMMATORY ACTIVITY OF 9α,11β-DICHLORO-17α,21-DIHYDROXY-$\Delta^{1,4}$-PREGNADIENE-3,20-DIONE-21-ACETATE (DD$\Delta^{1,4}$A) ADMINISTERED BY GAVAGE

Compound administered	Total dose, mg.	No. of rats	Mean thymus ratio ±S.E.	Mean dry weight of granuloma, mg. ±S.E.
0	0	10	3.94±0.17	42.5±4.3
Cortisol Acetate	1	10	2.96±0.20	34.2±2.4
	2	10	2.59±0.20	30.5±2.6
	4	10	2.17±0.10	29.3±2.9
	8	10	1.39±0.07	22.4±1.5
DD$\Delta^{1,4}$A	0.1	5	4.04±0.33	37.5±1.7
	0.5	6	4.20±0.30	35.2±5.5
	2.0	6	3.89±0.30	31.6±3.0

TABLE 2. THE THYMOLYTIC AND ANTI-INFLAMMATORY ACTIVITY OF 9α,11β-DICALORO-17α,21-DIHYDROXY-Δ^4-PREGNENE-3,20-DIONE-21-ACETATE (DDΔ^4A) ADMINISTERED BY GAVAGE

Compound administered	Total dose, mg.	No. of rats	Mean thymus ratio ±S.E.	Mean dry weight of granuloma, mg. ±S.E.
0	0	11	4.10±0.28	48.0±5.6
Cortisol Acetate	0.5	10	3.97±0.29	43.3±4.8
	1	10	3.14±0.19	34.6±3.2
	2	10	2.60±0.16	37.5±2.9
	4	10	2.15±0.12	27.9±1.8
	8	10	1.37±0.17	26.6±3.3
DDΔ^4A	0.5	4	4.65±0.26	39.4±4.8
	2	5	4.27±0.27	34.0±2.5
	8	4	4.14±0.33	46.6±7.6

basis of the thymolytic activity. DDΔ^4A, even at a dose of 8 mg., produced no involution of the thymus gland nor any consistent anti-inflammatory activity. This is to be contrasted with cortisol acetate which produced a statistically significant thymus involution at the one mg. dose (Table 2). Significant thymolytic and anti-inflammatory activity was observed when DDΔ^4A was injected subcutaneously at a total dose of 5 mg. (Table 3). In a second pellet granuloma test (performed by Dr. Elva G. Shipley) DDΔ^4A, DD$\Delta^{1,4}$A, and BFΔ^4A were less active than the standard cortisol (Table 4). One compound, DD$\Delta^{1,4}$A, was less than one-half as active as cortisol by a glycogenic assay (Table 5).

DISCUSSION

The relative potency of the three dihalo corticoids compared to cortisol are summarized in Table 6. DD$\Delta^{1,4}$A had an activity equal to or less than cortisol by the four different bioassays that were employed in this study. These results do not confirm the findings of Robinson *et al.* (1), who reported this steroid to be 16 times as active as cortisol by a granuloma pouch test. The corresponding 11β-hydroxy steroid, 9α-fluoroprednisolone, has been shown to be 9 to 28 times as active as cortisol (4, 5) which

TABLE 3. THE THYMOLYTIC AND ANTI-INFLAMMATORY ACTIVITY OF DDΔ^4A ADMINISTERED BY SUBCUTANEOUS INJECTION

Compound administered	Total dose, mg.	No. of rats	Mean thymus ratio ±S.E.	Mean dry weight of granuloma, mg. ±S.E.
0	0	7	4.84±0.21	26.1±2.9
Cortisol Acetate	0.5	9	3.89±0.16	20.4±1.7
	1	10	3.22±0.15	17.3±2.6
	2	10	1.44±0.19	17.7±1.6
	4	10	0.71±0.05	10.8±1.6
DDΔ^4A	5	10	3.51±0.20	21.4±2.7

TABLE 4. THE ANTI-INFLAMMATORY ACTIVITY OF DDΔ⁴A, DDΔ¹,⁴A, AND BFΔ⁴A ADMINISTERED BY INJECTION

Material administered	Total dose, mg.	No. of rats	Dry mean granuloma weight, mg. (range)
0	0	103	11.6±2.75*
Cortisol	0.5	5	9.8 (7.8–14.2)
	1.0	15	7.32 (5.0–11.0)
	2.0	5	5.8 (5.1– 6.6)
DDΔ⁴A	0.25	5	13.1 (10.8–16.8)
	1.0	5	10.6 (9.7–11.9)
DDΔ¹,⁴A	0.06	5	12.1 (9.4–14.9)
	0.25	5	11.1 (10.0–11.8)
	1.0	5	8.4 (6.5–10.2)
	2.0	5	7.8 (6.8– 8.3)
BFΔ⁴A	0.5	5	9.0 (7.1–10.6)
	2.0	5	10.1 (9.6–10.4)

* Standard Deviation.

TABLE 5. THE GLYCOGEN ACTIVITY OF DDΔ¹,⁴A ADMINISTERED BY INJECTION

Material administered	Total dose, mg.	No. of animals	Liver glycogen, mg./100 gm. B.W.
0	0	14	1.24 (0.80– 2.38)
Cortisol	0.05	4	5.00 (3.03– 7.50)
	0.1	4	11.75 (10.34–12.46)
DDΔ¹,⁴A	0.1	4	1.85 (1.62– 2.04)

TABLE 6. RELATIVE POTENCY OF 9α,11β-DIHALO CORTICOIDS

Steroid	Bioassay	Approximate relative potency cortisol = 1	Reference
DDΔ¹,⁴A	Rat Thymolytic Gavage	<0.5	This Report
	Rat Anti-Inflammatory Gavage	1.0	This Report
	Granuloma Pouch	16	Robinson *et al.*, 1959
	Rat Anti-Inflammatory Injection	0.5	This Report
	Rat Glycogenic Injection	0.5	This Report
DDΔ⁴A	Rat Thymolytic Gavage	<0.13	This Report
	Rat Thymolytic Injection	0.13	This Report
	Rat Anti-Inflammatory Injection	0.1	This Report
BFΔ⁴A	Rat Anti-Inflammatory Injection	<0.25	This Report

means that the replacement of the 11β-hydroxy group by the 11β-chloro group resulted in at least a ten-fold reduction in activity. Similar reduction in activity for DDΔ^4A and BFΔ^4A are indicated.

REFERENCES

1. Robinson, C. H., L. Finckenor, E. P. Oliveto and D. Gould: *J. Am. Chem. Soc.* **81:** 2191. 1959.
2. Robert, A. and J. E. Nezamis: *Acta Endocrinologica* **25:** 105. 1957.
3. Chart, J. J., E. G. Shipley and R. K. Meyer: *Proc. Soc. Exptl. Biol. Med.* **90:** 127. 1955.
4. Dorfman, R. I.: Unpublished data.
5. Fried, J. and A. Borman: *Vitamins and Hormones* **16:** 368. 1958.

TISSUE RETENTION OF CORTISOL AND METABOLITES INDUCED BY ACTH: AN EXTRA-ADRENAL EFFECT[1]

DAVID L. BERLINER, NANCY KELLER[2] AND THOMAS F. DOUGHERTY

Department of Anatomy, Division of Radiobiology, University of Utah College of Medicine, Salt Lake City, Utah

ABSTRACT

ACTH, both exogenously administered and endogenously produced, causes a retention of cortisol and its metabolites in blood, kidney, thymus and liver of adrenalectomized mice. Exogenous ACTH causes an inhibition of conjugation of corticoid metabolites. Following administration of cortisol-4-C^{14}, these radioactive compounds were found in the livers of normal and adrenalectomized mice (ACTH-treated and non-treated): tetrahydrocortisol; tetrahydrocortisone; Reichstein's substance "E"; the 20α epimer of Reichstein's substance "E"; Reichstein's substance "U"; and cortisone. Remaining unidentified were several compounds other than those listed whose structures were established.

ACTH infusion (1) and surgical stress (2, 3, 4) have been shown to raise the levels of 17-hydroxycorticosteroids (17-OHCS) in peripheral blood. It is now becoming evident that the mechanism of ACTH action may not be a simple, uncomplicated stimulation of the adrenal cortex, but may also involve an influence on some factor or factors controlling the rate at which corticoids and their metabolites are removed from blood and tissue. Firschein, *et al.* (5) demonstrated not only increased amount of radioactivity in the blood and tissues of stressed rats injected with cortisol-4-C^{14}, but also a decrease in the rate of disappearance of radioactivity from blood and tissue. Elevated levels of 17-OHCS have been observed in dying patients coincidentally with a very prolonged half-life of free plasma 17-OHCS and in one patient decreased glucuronide conjugate formation (6). A study by Englert, *et al.* (7) implicated the liver in the control of plasma concentrations of 17-OHCS. *In vitro* studies from this laboratory (8) demonstrated a decreased capacity of livers from stressed mice to conjugate steroid metabolites when incubated with cortisol-4-C^{14}; inhibition of conjugation was again observed when livers of ACTH-treated, adrenalectomized mice were incubated with the radioactive cortisol.

Received July 12, 1960.

[1] This work has been supported by grants from the U. S. Public Health Service (Cooperative Leukemia—#34-5917) and the American Cancer Society (#38-5202).

[2] This work has been done in partial fulfillment of the requirements of the University of Utah for the Ph.D. degree.

The experiments to be described in this paper were carried out in whole animals, two groups of which were adrenalectomized in order to demonstrate the extra-adrenal character of some ACTH effects.

MATERIAL AND METHODS

Tissues studied in these experiments were taken from adrenalectomized and intact female mice of the AK strain. The average age of the animals at the time of sacrifice was 74 days.

Those mice which were adrenalectomized were allowed to recover from any effects of the operation for a 4- to 7-day period, at the end of which 14 mice received 1 I.U. (International Unit) of α-corticotrophin[3] (ACTH preparation used) per day for 5 days (cumulative dose = 5 I.U.) and another group of 12 adrenalectomized mice were injected with an 0.9% saline solution. The 18 intact animals of the control groups also got a saline injection. The injections were 0.25 ml. in volume and were administered intraperitoneally.

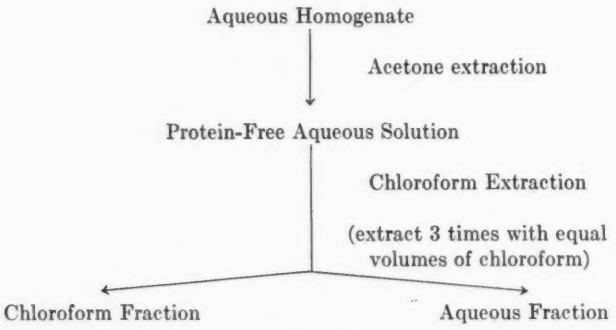

FIG. 1. Procedure for liver tissue fractionation. To separate steroids from protein, acetone is added to the aqueous homogenate, the temperature of this mixture is raised to 45° C, and then the mixture is filtered through a filter paper. The filtrate of acetone and water is freed of acetone by evaporation under partial vacuum to produce the protein-free aqueous solution.

After the last injection of ACTH or saline, 2 to 3 hours were allowed to elapse before a tail vein injection of cortisol-4-C^{14} was given. Then the animals were sacrificed in groups of 2 to 6 mice, at 3 min., 7 min., 15 min., 50 min., and 100 min. after administration of the radioactive steroid.

The mice were killed by spinal cord severance. Immediately after death, blood samples were withdrawn from the heart. Then in rapid succession livers, lymph nodes (axillary and inguinal), spleens, kidneys, and thymi were removed. After the organs were weighed, they were immediately frozen in a deep freeze (−12° C) and kept frozen until homogenized.

Aqueous homogenates of all tissues (except the blood) were prepared. The liver homogenates were fractionated according to the scheme given in Figure 1.

Aliquots of blood, homogenates and $CHCl_3$ and H_2O fractions from the liver were plated and counted in a gas flow thin window counter operating in the Geiger range. The counts per minute (CPM) above background were corrected for mass absorption.

The measurements of amount of radioactivity in the blood and in the aqueous

[3] Potency 100 I.U./mg.

homogenates of liver, thymus and kidneys were submitted to statistical analysis. First, the data were expressed as CPM/mg. of dry tissue (relative specific activity) as a function of time. Zero time equalled the time of cortisol-4-C^{14} injection. The curves for the disappearance of radioactivity from these tissues could be described by the expected exponential functions, so that the equations for the straight lines of the semi-logarithmic plots were of the form $y = ae^{bx}$, where:

y = CPM/mg. of tissue dry weight
x = time in minutes
a = the ordinate intercept
b = the slope of the line

Each disappearance curve was fitted to the experimental data by the method of least squares.[4] By this method, the ordinate intercept and the slope of the line can be computed. The biological half-life can also be computed from the statistically derived equation. Regression coefficients were tested for significant differences between the groups of animals by the "t" test.

Total CPM in both chloroform and water fractions were determined and ratios were taken between the activity which was present in chloroform and that in water at the various time intervals. These ratios represented the chloroform soluble compounds vs. water soluble conjugates in the liver.

Decrease of the ratio with time was a power function which means that $y = ax^b$ where:

y = CPM as chloroform soluble compounds/CPM as water soluble compounds
x = time in minutes
a = the ordinate intercept
b = the slope of the line

The ratios were plotted on graph paper on which both the ordinate and the abscissa are marked off in logarithmic scales. Then the curves were fitted to the experimental data by the method of least squares. Analysis of variance was used to test for significant differences between the slopes of the curves and between their ordinate intercepts.

After aliquots of the chloroform fraction were plated, the remaining portion of each sample was prepared for chromatography by the standard method. Zaffaroni chromatographic systems were used throughout (9).

To remove fat, the samples were overrun (mobile phase allowed to drip from the paper) in hexane-formamide for several hours. Preliminary separation of the free steroids from the liver of each animal was accomplished by an overnight run in chloroform-formamide. The mobile phase solvent which dropped from the end of the papers during this procedure was collected in beakers and later pooled for chromatographic analysis. Those steroids remaining on the paper were usually separated into four major zones (Fig. 2). The latter were traced out by the strip scanning Geiger counter as previously described (10). A typical tracing which shows the zones into which the chloroform fraction separated and also the relative position of each zone on the paper is presented in Figure 2.

After addition of known standard solutions (non-radioactive) of suspected compounds, pools of the major zones were chromatographed to separate the components. The radioactive spots on the paper chromatogram were again traced out by the strip scanning equipment. (This procedure was routine after every chromatographic run.) Then the strips were examined under ultraviolet light to detect any U. V. absorption

[4] The principles of this method are described in *Analysis of Straight-Line Data* by F. S. Acton (J. Wiley & Sons, New York, 1959). For an example of the adaptation of this method to a power function see: Stover, B. J., D. R. Atherton and N. Keller: *Radiation Research.* **10:** 130. 1959.

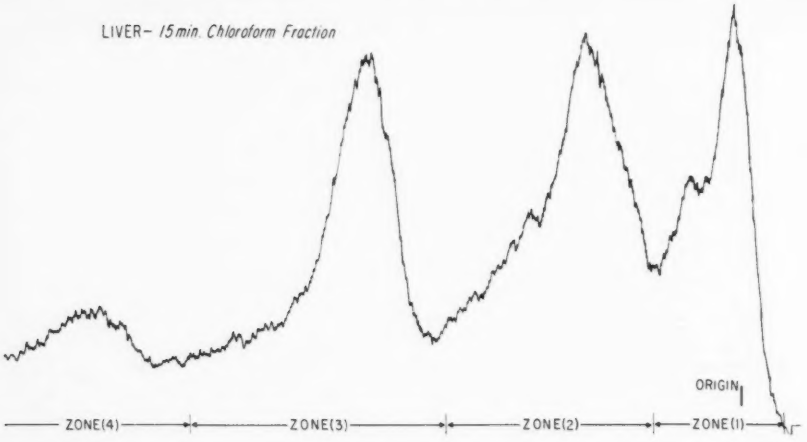

FIG. 2. Tracing from strip counter showing radioactive zones found after initial chromatography of a 15 minute liver chloroform fraction.

spots, and a TPTZ color test (9) was done when applicable. Acetylated and oxidized derivatives of the detected radioactive steroids were prepared, using acetic anhydride and chromic trioxide (11). Acetates were run in benzene-formamide and oxidized derivatives in benzene-formamide or hexane-benzene-formamide.

Positive identification of a radioactive compound from the liver was established by 1) constant association of a radioactive peak with its U. V. absorption spot and/or its positive triphenyltetrazolium (TPTZ) reaction for the a-ketol side chain and more important, 2) the degree to which it ran consistently with the added standard steroid through chemical modifications (oxidation and acetylation) and changes of mobile phase in chromatography.

The cortisol-4-C^{14} had a specific activity of 4.21 millicuries per millimole. Since 100,000 c.p.m. were administered, each mouse received 3.16×10^{-2} μmoles of cortisol. The cortisol was injected in a volume of 0.25 ml. of which 0.20 was saline and 0.05 ml. was alcohol.

RESULTS

Disappearance of Radioactivity (as CPM/mg. of dry wt.) from Blood and Tissue Homogenates

The semilogarithmic plots of the blood radioactivity disappearance curves show that adrenalectomized animals treated either with saline or ACTH have consistently higher relative specific activities in blood than do intact controls; the ACTH animals have the highest levels. However, biological half life of injected radioactivity in blood is not significantly altered by the presence or absence of the adrenals, nor by the pretreatment of the animals with ACTH, (Table 1), since the regression coefficients do not differ significantly from one group to another.

One of the major target organs for the lymphocytokaryorrhectic effects of adrenal steroids like cortisol is the thymus. In this tissue, regression coefficients for disappearance of radioactivity in thymi of intact control

TABLE 1. EQUATIONS OF LINES REPRESENTING DISAPPEARANCE OF RADIOACTIVITY FROM BLOOD, THYMUS, AND LIVER

Tissue	Group of animals	Equation	Biological half life (min.)
Blood	Intact Controls	$Y = 26.5e^{-0.0256x}$	28.6
	Adrenalectomized + Saline	$Y = 40.6e^{-0.0248x}$	27.9
	Adrenalectomized + ACTH	$Y = 50.6e^{-0.0233x}$	29.8
Thymus	Intact Controls	$Y = 16.9e^{-0.0270x}$	25.8
	Adrenalectomized + Saline	$Y = 22.4e^{-0.0251x}$	27.6
	Adrenalectomized + ACTH	$Y = 23.5e^{-0.0189x}$	36.6
Liver	Intact Controls	$Y = 116e^{-0.0197x}$	35.3
	Adrenalectomized + Saline	$Y = 133e^{-0.0201x}$	34.0
	Adrenalectomized + ACTH	$Y = 118e^{-0.0135x}$	51.5

animals and the adrenalectomized—saline group are significantly different from the regression coefficient found for the adrenalectomized ACTH-treated group of mice ($P < 0.01$). The amounts of radioactive compounds are highest and decrease at the slowest rate in the adrenalectomized ACTH-treated mice (Table 1).

A semi-logarithmic plot of the liver relative specific activities vs. time shows a pattern similar to that for the thymus data, i.e., the regression coefficients and hence the biological half lives for the disappearance curves do not differ significantly between normal, intact animals and those adrenalectomized and treated with saline, although regression coefficient and biological half life are different ($P < 0.01$) for the adrenalectomized ACTH-treated animals. Relative specific activity in livers of adrenalectomized ACTH-treated mice decreases at a rate which is only about 67% of the rate of decrease found for the other two groups of mice (Table 1, Fig. 3).

The disappearance of radioactivity vs. time for the kidney data shows that again as in blood, thymus and liver, the ACTH-treated, adrenalectomized mice maintain the highest levels of radioactive steroids over the measured time period (Fig. 4). Also, the regression coefficients and hence biological half lives, were significantly different ($P < 0.01$) between adrenalectomized ACTH-treated and the intact non-treated controls. There was no significant difference between the regression coefficients of the two adrenalectomized groups, however, demonstrating that apparently, in the absence of the adrenal, pretreatment with ACTH causes no further decrease in the rate at which steroids can be removed from the kidney.

Ratios of Chloroform Extractable Compounds/Conjugated Steroids in the Liver

The decrease with time in the ratios of chloroform extractable steroids to conjugates in the liver occurred at roughly the same rates in all three groups of animals, since analysis of variance showed that the slopes of the three lines were not significantly different. However, the ordinate intercept of the line representing the decrease of the ratio in the ACTH-

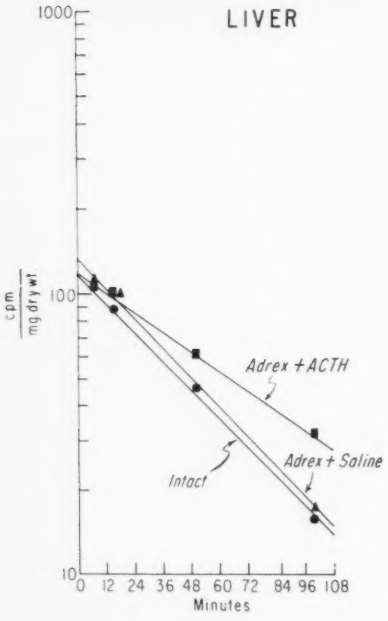

FIG. 3. Regression lines for relative specific activities in the liver, after cortisol-4-C^{14} infusion.

treated group is significantly greater ($F = 54.4$) than the ordinate intercepts for the data from the other two groups. To summarize, the ratios of the chloroform extractable compounds to conjugated steroids are consistently higher in those animals which recieved ACTH, although the ratios decrease with time at a similar rate in all three groups (Table 2).

Radioactivity in the Liver. Per cent of injected radioactivity in homogenates of whole liver is shown in Table 3, and Figure 5 shows the changes in concentration of conjugates in the liver as a function of time. During

KIDNEYS

GROUP OF ANIMALS	EQUATION OF LINE	BIOLOGICAL HALF LIFE (min)
Intact Controls	$y = 91.8e^{-0.0252x}$	27.5
Adrex + Saline	$y = 93.7e^{-0.0198x}$	35.0
Adrex + ACTH	$y = 118e^{-0.0200x}$	34.8

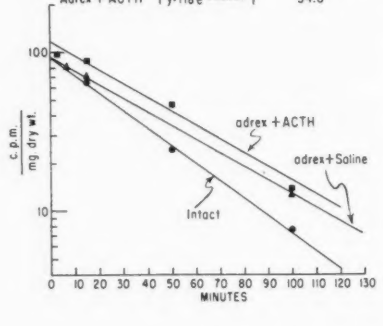

FIG. 4. Regression lines and their equations for relative specific activity in the kidney.

TABLE 2. EQUATIONS OF LINES REPRESENTING THE DECREASE OF THE RATIO BETWEEN FREE STEROIDS AND WATER SOLUBLE CONJUGATES IN THE LIVER

Group of animals	Equation
Intact Controls	$Y = 24.0X^{-0.5138}$
Adrenalectomized + Saline	$Y = 22.4X^{-0.4727}$
Adrenalectomized + ACTH	$Y = 52.9X^{-0.5534}$

TABLE 3

Time after cortisol-4-C^{14} inject.	% of injected dose in whole liver		% of radioactivity in whole liver as free cortisol	
	Intact controls	Adrex + ACTH	Intact controls	Adrex + ACTH
3 min.	30.8	29.0	14.9	6.5
7 min.	37.3	33.3	14.4	5.3
15 min.	33.3	39.9	7.6	4.0
50 min.	18.0	22.0	3.6	2.2
100 min.	5.94	14.2	—	—

the 3- to 15-minute period the livers of both adrenalectomized, ACTH-treated animals and normals contain about one-third of the injected radioactivity, with the livers of the ACTH group reaching a high of about 40% of injected radioactivity at 15 minutes. At three minutes, the normal animals have two to three times as much conjugate in their livers as do adrenalectomized animals and it is not until 50 minutes that the concentration of conjugates becomes equal in the various groups of animals.

Table 3 also shows that the amount of free cortisol (as determined by

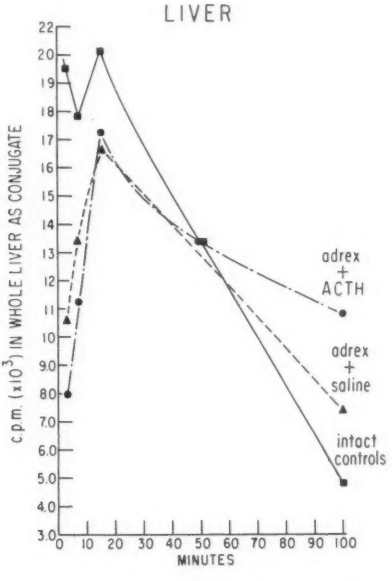

FIG. 5. Concentration of conjugated steroid in livers of mice as a function of time.

chromatography) in the livers of the adrenalectomized, ACTH-treated animals is considerably less than in livers of the normals.

The method of identification of radioactive steroids from liver by paper chromatography was outlined above and the preparation of homogenate for chromatography was shown in Figure 1, while Figure 2 shows the separation of the beginning material into the four major zones.

Zone (1) contained the most highly polar compounds, which could have been the cortols, cortolones, and 6β-hydroxy compounds. β-cortol was available and 100 μg of this compound were added to zone 1, which was then overrun in chloroform-formamide for 4 days. Utilizing a method described by Cox (12), it was found that β-cortol had not separated from the other highly polar compounds at the origin. After acetylation, the whole origin area was rerun in chloroform-formamide to the front and was thereby separated into 3 areas. Two peaks occurred at or near the origin, while the third ran with the solvent front (similar to a triacetate).

Zone (2) contained four cortisol metabolites, all of which were identified. These metabolites were:

1. Tetrahydrocortisol
2. Reichstein's substance E(4-pregnene-11β,17α,20β,21-tetrol-3-one)
3. The 20α epimer of Reichstein's substance E
4. Tetrahydrocortisone

Zone (2) was acetylated to separate these four metabolites.

Tetrahydrocortisol. Tetrahydrocortisol was acetylated to tetrahydrocortisol-3,21-diacetate, which was then oxidized to tetrahydrocortisone-3,21-diacetate and rechromatographed.

Reichstein's Substance E. Reichstein's substance E was acetylated to Reichstein's E-20,21-diacetate, which was then oxidized to Reichstein's U-20,21-diacetate (4-pregnene-17α-ol-3,11-dione-20β-21-diacetate) and rechromatographed.

Reichstein's Substance E, 20α. Acetylation of Reichstein's substance E, 20α formed the diacetate. Oxidation yielded Reichstein's U-20α,21-diacetate which was rechromatographed.

Tetrahydrocortisone. Tetrahydrocortisone was acetylated to the 3,21-diacetate derivative and the chromatographic run carried out. The diacetate was oxidized and rechromatographed in a different system.

Zone (3) contained the unmetabolized cortisol. After some cortisol standard was added to zone 3 it was run in chloroform-formamide to the front. Subsequently the cortisol was acetylated to the 21-monoacetate and rechromatographed in another system.

Zone (4) was identified as substance U of Reichstein. Chromatography of unknown and standard in chloroform-formamide to the front, then acetylation to Reichstein's U 20,21-diacetate and rechromatography in benzene-formamide to the front demonstrated that the radioactive peak did not separate from the added standard.

The pool of mobile phase solvent which dripped from the strips during the separation of chloroform fraction (Fig. 1) into the four major zones (Fig. 2) was found to contain cortisone. Standard cortisone was added to the pooled solvent which was then run in chloroform-formamide to the front, yielding a radioactive peak associated with the added standard. After elution, the radioactive area was divided into two portions. One portion was oxidized, giving 4-androstene-3,11,17-trione which was chromatographed in benzene-formamide to the front and the other portion was acetylated to the 21-monoacetate of cortisone, then overrun in benzene-formamide for 3 hours. All these procedures indicated that the unknown was a single compound which ran in all cases like the added standard.
Compounds identified:

Zone (1) very highly polar compounds, not completely identified, probably of the cortol and cortolone type, or 6β-hydroxy compounds.
Zone (2) tetrahydrocortisol
Reichstein's E
Reichstein's E, 20α
tetrahydrocortisone
Zone (3) cortisol
Zone (4) substance U of Reichstein
Pooled solvent which dripped from the strips: cortisone

The relative amounts of each of the identified cortisol metabolites and the amounts of conjugate as glucuronide are in the process of being determined.

DISCUSSION

Radioactivity in the blood

ACTH and stress have been shown to raise the levels of plasma 17-OHCS (measured as Porter-Silber chromagen, i.e. steroids with a dihydroxy ketone side chain at C-17) and to delay the disappearance of 17-OHCS from the plasma. Kuipers, *et al.* (13) gave 50 mg. of cortisol intravenously to dogs and found that a subsequent injection of 5 I.U. of ACTH produced a marked prolongation in half-life of plasma 17-OHCS. Firschein, *et al.* (5) stressed rats, then injected cortisol-4-C^{14} one hour later. Semilogarithmic plots of their data as CPM/gm. of plasma dry weight vs. time showed that radioactive steroid disappeared more slowly from plasma of stressed animals than from normal plasma. Dougherty and Berliner (14) demonstrated that in adrenalectomized mice given 5 I.U. of ACTH immediately followed by cortisol injection, that the half-life of radioactive steroid and its metabolites was increased over that of control, adrenalectomized animals.

Our experiments showed an increased level of blood radioactivity under

the influence of ACTH. The half-life of radioactive compounds was not lengthened in the two adrenalectomized groups as compared to the normal, intact control group, but it should be kept in mind that the animals which received ACTH in these experiments were pretreated with corticotropin for 5 days before they were injected with the radioactive steroid.

In the absence of the adrenals, an increase of the administered steroid in blood must be explained by some action of ACTH on extra-adrenal tissues. Previous work has demonstrated the ability of extra-hepatic tissue to metabolize cortisol. Cultured uterine fibroblasts grown on a medium containing cortisol-4-C^{14} could form Reichstein's substance E, Reichstein's substance U, and possibly 4-androstene-11β-ol-3,17-dione (15). Eviscerated rats with ligated renal vessels could convert cortisol-4-C^{14} to Reichstein's substance E, Reichstein's substance U, dihydrocortisol and perhaps 4-androstene-11β-ol-3,17-dione (16). ACTH may be inhibiting the enzymes which carry out these steroid conversions either by directly inhibiting the enzymes or by limiting the amounts and/or availability of cofactors present.

Radioactive compounds in the liver

Our results describe a higher than normal content of radioactive compounds in the liver of both adrenalectomized groups and furthermore radioactivity disappeared much more slowly from the livers of those animals which were ACTH-treated. Amounts of metabolite in livers of ACTH-treated mice are greater than the amounts found in livers of intact controls (Table 3), but the intact controls convert more metabolite to conjugate since cholorform soluble compounds/water soluble compounds was always significantly lower for livers from intact controls than for livers from ACTH-treated adrenalectomized mice.

Other workers have shown that the liver can influence blood level and rate of removal of 17-OHCS from blood. Englert, *et al.* (7) found that tetrahydrocortisone was more readily conjugated than tetrahydrocortisol by human liver. Gold, *et al.* (17) observed that the urinary ratio of tetrahydrocortisol to tetrahydrocortisone is raised when humans are stressed or injected with ACTH. ACTH may be reducing the quantity of conjugated compounds in our animals by a mechanism favoring the production of tetrahydrocortisol over that of tetrahydrocortisone.

Recirculation of unconjugated metabolites might account for the high blood levels of radioactive compounds. It has been demonstrated by Weichselbaum and Margraf (18) that even tetrahydrocortisone can exist in normal blood as the free (unconjugated) compound.

In vitro studies (to be published) with liver from adrenalectomized and ACTH-treated rats and mice demonstrated the inhibition of conjugation of cortisol, corticosterone and tetrahydro 11 keto-progesterone by ACTH. Since tetrahydro 11 keto-progesterone is a ring A reduced compound known

to undergo conjugation, it would appear that conjugation is inhibited because the tetrahydro compound cannot couple with the acid component as readily as when ACTH is absent. However, the possibility is not eliminated that in the whole animal the transformation to the dihydro compound is lessened under the influence of ACTH, although no dihydro compounds were identified as metabolites of cortisol in the free steroid fraction of mouse liver in our experiments.

Another possible explanation for reduced conjugate formation could be given by Reid (19), who reported that 2 to 3 weeks after adrenalectomy the uridine nucleotides, including UDPGA are decreased in rat liver. At this time endogenous ACTH is high (20).

Radioactivity in the kidney

The relative specific activity of kidney followed the same general pattern seen in blood, thymus and liver—the adrenalectomized, ACTH-treated animals had the highest relative specific activities followed by adrenalectomized, saline-treated animals, and the intact controls showed the lowest CPM/mg. of tissue dry weight. In the kidney, however, unlike the thymus and the liver, the regression coefficients were not different for adrenalectomized, ACTH-treated and adrenalectomized, saline-treated but both adrenalectomized groups lost radioactivity from kidneys at a rate significantly lower than that for normal controls. These data indicate that at least under our experimental conditions the endogenous ACTH present in the adrenalectomized saline-treated mice was sufficient to lower to less than normal the rate at which relative specific activity decreased in kidney tissue, since pretreatment of the other group of adrenalectomized animals with ACTH did not further reduce the rate of decrease of relative specific activity. Sandberg and Slaunwhite (21) have suggested that in Addisonians the higher plasma radioactivity found after cortisol-4-C^{14} injection may be due to slower excretion of steroids by these patients, due to reduced glomerular filtration of the glucuronide fraction, and thus it may be that high endogenous levels of ACTH have some limited effect on steroid excretion by the kidney.

Ganis, *et al.* (22) have reported on the metabolism of cortisol by bovine kidney homogenates. These authors identified Reichstein's substance E, 4-androstene-11β-ol-3,17-dione, 4-androstene-3,11,17-trione, and cortisone in the free steroid fraction. Unpublished observations from our laboratory show that kidney minces are capable of forming glucuronides from tetrahydro corticoid metabolites. It could be that ACTH is influencing metabolism in kidney as well as thymus and liver. The liver seems to be the organ where ACTH could have the greatest effect on steroid metabolism however, since it appears to be capable of carrying out a greater variety of steroid interconversions and Nose and Lipmann (23) have shown that kidney and intestine do not contain steroid sulfokinase, while the liver does.

Blair and Morgen, as quoted by other workers (24) have observed a de-

crease in the conjugation of steroids in adrenalectomized patients after ACTH administration.

Other extra-adrenal effects of ACTH have been demonstrated: lipolytic (25, 26); accumulation of cholesterol in reticuloendothelial cells (27); and nitrogen retaining effect (28).

REFERENCES

1. Nelson, D. H., L. T. Samuels, D. G. Willardson and F. H. Tyler.: *J. Clin. Endocrinol.* **11:** 1021. 1951.
2. Cope, C. L. and B. Hurlock: *Clinical Sci.* **13:** 69. 1954.
3. Weichselbaum, T. E., H. W. Margraf and R. Elman: *Fed. Proc.* **11:** 287. 1953.
4. Moore, F. D.: *Rec. Prog. Hormone Res.* **13:** 511. 1957.
5. Firschein, H. E., F. De Venuto, W. Fitch, E. Pearce and U. Westphal: *Endocrinology* **60:** 347. 1957.
6. Sandberg, A. A., K. Eik-Nes, C. J. Migeon and L. T. Samuels.: *J. Clin. Endocrinol. and Metab.* **16:** 1001. 1956.
7. Englert, E., H. Brown, S. Wallach and E. L. Simons: *J. Clin. Endocrinol. and Metab.* **17:** 1395. 1957.
8. Berliner, D. L., C. J. Nabors, Jr. and T. F. Dougherty: 41st Meeting of the Endocrine Society, 1959.
9. Zaffaroni, A.: *Rec. Prog. in Hormone Res.*, **8:** 51. 1953.
10. Berliner, D. L., O. V. Dominguez and G. Westenskow: *Analytical Chem.* **29:** 1797. 1957.
11. Berliner, D. L. and H. A. Salhanick: *Analytical Chem.* **28:** 1608. 1956.
12. Cox, R. I.: *J. Biol. Chem.* **234:** 1693. 1959.
13. Kuipers, F. K., R. S. Ely and V. C. Kelly: *Endocrinology* **62:** 64. 1958.
14. Dougherty, T. F. and D. L. Berliner: Symposium on Liver Function. Publication No. 4 of the Am. Inst. of Biol. Sci. 416. 1958.
15. Sweat, M. L., B. I. Grosser, D. L. Berliner, H. E. Swim, C. J. Nabors, Jr. and T. F. Dougherty: *Biochim. Biophys. Acta* **28:** 591. 1958.
16. Berliner, D. L., B. I. Grosser and T. F. Dougherty: *Arch. Biochem. Biophys.* **77:** 81. 1958.
17. Gold, N. I., E. Singleton, D. A. Macfarlane and F. D. Moore: *J. Clin. Invest.* **37:** 813. 1958.
18. Weichselbaum, T. E. and H. W. Margraf: *J. Clin. Endocrinol. and Metab.* **19:** 1011. 1959.
19. Reid, E.: Proceedings of the Brook Lodge Symposium (Upjohn). p. 477. 1958.
20. Hodges, J. R. and J. Vernikos: *J. Physiol.* **150:** 683. 1960.
21. Sandberg, A. A. and W. R. Slaunwhite, Jr.: *J. Clin. Endocrinol. and Metab.* **18:** 662. 1958.
22. Ganis, F. M., L. R. Axelrod and L. L. Miller: *J. Biol. Chem.* **218:** 841. 1958.
23. Nose, Y. and F. Lipmann: *J. Biol. Chem.* **233:** 1348. 1958.
24. Blair, A. J. and Morgen, R. O. cited by Dyrenfurth, I., J. C. Beck and E. H. Venning: *J. Clin. Endocrinol. and Metab.* **20:** 751. 1960.
25. White, J. E. and Engel, F. L.: *J. Clin. Invest.* **37:** 1556. 1958.
26. Schotz, M. C., G. M. C. Masson and I. H. Page: *Proc. Soc. Exper. Biol. & Med.* **101:** 159. 1959.
27. Dougherty, T. F. and D. L. Berliner: In Connective Tissue, Thrombosis, and Atherosclerosis. Ed.: I. Page. Acad. Press Inc., New York, N. Y. p. 143. 1959.
28. Engel, F. L. and J. Fredericks: *Endocrinology* **64:** 409. 1959.

ESTROGEN ANTAGONISMS: THE EFFECTS OF VARIOUS STEROIDS ON ESTRONE-INDUCED UTERINE GROWTH IN SPAYED RATS

RICHARD A. EDGREN[1] AND DAVID W. CALHOUN

Division of Biological Research, G. D. Searle and Co., Chicago, Illinois

ABSTRACT

Progesterone inhibits the uterine growth produced by estrone in rats spayed at 30 days of age; this antagonism is apparent only at intermediate response levels, produced by estrone at doses of 0.3 to 3.0 μg. DCA only inhibited the response produced by 3 μg of estrone. Previous studies also indicated that the uterine response produced by estrone at 1–3 μg was most sensitive to blockage by testosterone or estriol. This appears to be an example of other steroid hormones acting as estrone "buffers" at critical physiological levels. The uterine growth produced by 2 μg of estrone in rats spayed at 100 days of age was inhibited by progesterone and DCA, unaffected by cortisone acetate, and augmented by estriol and testosterone propionate.

OVER the past several years we have tried to gain insight into the problem of hormonal interactions by studying mixtures of an estrogen with representative natural steroids that might be involved with estrogens in the control of sexual development and cycles. Generally estrone was employed and the modification of its effects by the simultaneous administration of other steroids was examined. The effects on uterine growth in spayed rats of estrone and either estriol or 16-epiestriol (1) and the three primary natural estrogens (estrone, estradiol-17β or estriol) and testosterone propionate were described (2, 3). Also the activities of mixtures of estriol and progesterone have been examined (4). The effects of progesterone and desoxycorticosterone acetate (DCA) on the uterine growth stimulated by estrone in rats spayed prepuberally are described here, as well as the results of an experiment that examined the effects of various steroids on uterine growth of rats spayed as adults. The latter experiment was designed to resolve certain conflicts between results reported from this laboratory and those described by Velardo and his associates (5, 6). In a consideration of possible causes of these differences, age of rats at spaying seemed critical (3).

MATERIAL AND METHODS

None of the methods employed here differed significantly from those of earlier studies. Sprague-Dawley rats were spayed at 30 or 100 days of age and allowed about 10

Received August 11, 1960.

[1] Present address: Research and Development Divisions, Wyeth Laboratories, Philadelphia, Pennsylvania.

days for recovery from the operation and for uterine regression after which treatment was begun. All injections were subcutaneous, with dosages contained in 0.1 ml of corn oil. All animals were treated for three days and killed with chloroform 24 hours after the final injection. At autopsy the uteri were removed, cleaned and weighed wet on a torsion balance.

Logarithms of the uterine weights were used for statistical analyses. Variability in this test is roughly proportional to the mean of each group (1) and Tukey's test for non-additivity (7) showed the log transformation to be more satisfactory than the uterine weight itself. An analysis of variance was carried out for each experiment and the results used to compute least significant differences (at the 5% level).

RESULTS

Estrone-Progesterone Mixtures. In this experiment a dose-response curve for estrone was obtained by evaluating its uterine growth effects at doses of 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 μg. Simultaneously, the same doses were studied in combination with progesterone at 100, 1000 or 3000 μg. The progesterone doses were also tested alone and an oil control group was included. These 28 groups, of four rats each, formed a single unit of the experiment and three replicates were performed, making a total of 12 rats at each dose level or combination. Variability in uterine weight of these rats was estimated from the following analysis of variance (Anova):

Term	Degrees of freedom	Mean square	F
Between Weeks	2	0.0019	
Treatments	27	0.4715	28.2 =0.4715/0.0167
Interaction	54	0.0167 $=s^2$	3.04 =0.0167/0.0055
Residual	238	0.0055	

From this analysis and the estimated standard deviation ($s = \pm 0.129$ log units) the least significant difference between means of 12 rats was estimated as ± 0.105 log units. According to this criterion the mean responses of several groups that received mixtures of estrone and progesterone were significantly smaller than means for comparable estrone-treated groups. One hundred micrograms of progesterone significantly depressed the response to 0.3, 1.0, and 3.0 μg of estrone; 1000 μg of progesterone depressed the response to 1 and 10 μg but not to 3 μg of estrone (Fig. 1). Three thousand micrograms of progesterone were not inhibitory. Thus low to moderate doses of progesterone will depress the response to estrone at doses that produce intermediate levels of uterine growth.

Estrone-DCA. Estrone again was studied at doses of 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 μg both by itself and in combination with DCA at 10, 100, or 1000 μg. The DCA itself was tested at these three doses and an oil-treated group was included. Each group contained four animals and four identical replicates completed the design. Variability was estimated from the following Anova based upon the log transformation:

Term	Degrees of freedom	Mean square	F
Between Weeks	3	0.0792	
Treatments	27	0.8462	114.4 = 0.8462/0.0074
Interaction	81	0.0074 = s^2	1.68 = 0.0074/0.0044
Residual	336	0.0044	

The standard deviation was estimated as ± 0.086 log units and the least significant difference between means of 16 rats was estimated as ± 0.0598 log units. The point representing estrone at 3 μg plus DCA at 1000 μg was significantly lower than the estrone-alone point for 3 μg (Fig. 2). However, since 18 points were compared to the estrone dose-response curve, it is not unlikely that one point should have deviated significantly from the standard value.

Estrone Plus Various Steroids in Older Rats. Variation in this experiment was estimated by the following Anova:

Term	Degrees of freedom	Mean square	F
Treatments	16	0.1463	33.5
Residual	104	0.0043 = s^2	

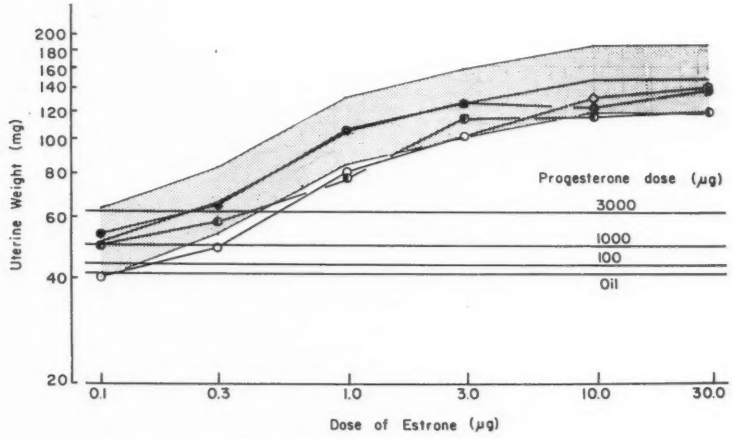

FIG. 1. The effects of combinations of estrone and progesterone on the uterine growth of spayed, immature rats. The line bordered by the stippled zone indicates the responses of rats to estrone alone, and the stippled area represents the "least significant difference" band for this dose-response curve. The circles indicate the responses to mixtures of estrone and progesterone at 100 μg; half-circles, estrone and progesterone at 1000 μg; dots, estrone and progesterone at 3000 μg. The horizontal lines indicate responses to oil control and groups of rats that received progesterone at 100, 1000 or 3000 μg. Each point, including those along the estrone-alone curve and those for oil and progesterone, is the mean of 3 groups of 4 rats each or a total of 12 rats. Any point falling outside of the stippled zone represents a response that is significantly different (at the 5% level) from that to estrone alone at the same dose. Both ordinate and abscissa are log scales.

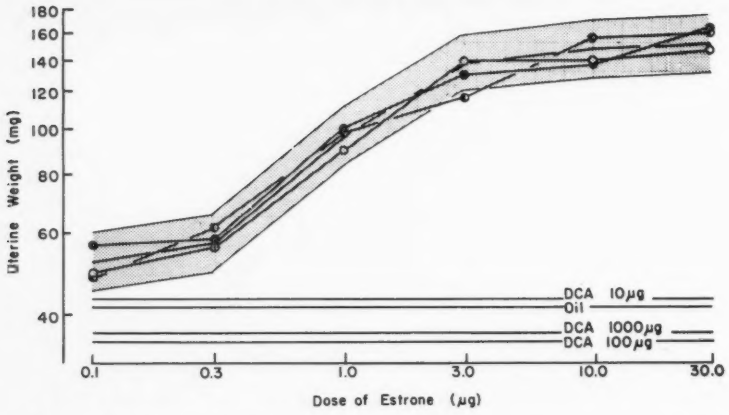

FIG. 2. The effects of combinations of estrone and desoxycorticosterone acetate (DCA) upon uterine growth of spayed, immature rats. For details see caption for Fig. 1. The circles indicate the responses to mixtures of estrone and DCA at 10 μg; half-circles, estrone and DCA at 100 μg; and dots, estrone and DCA at 1000 μg. Each point is the mean of 4 groups of 4 rats each or a total of 16 rats. Any point falling outside of the stippled area represents a response that is significantly different (5%) from estrone alone at the same dose.

From the calculated standard deviation ($s = \pm 0.066$ log units) the least significant difference between means was estimated as approximately ± 0.067 log units. Two micrograms of estrone produced slight, but significant growth of the uterus. The addition of testosterone propionate produced significant augmentation at doses of 300 and 1000 μg, whereas, 100 μg had no effect. Cortisone acetate (300–3000 μg) had no effect. Progeste-

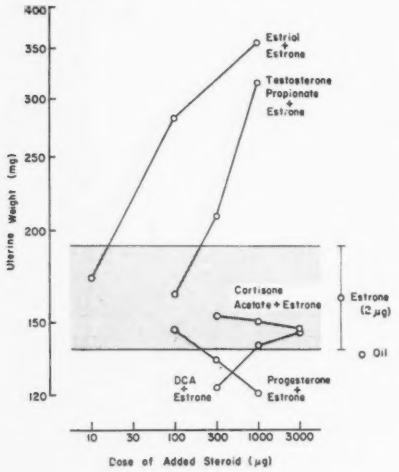

FIG. 3. The effects of various steroid hormones on estrone-stimulated uterine growth in rats that had been spayed at 100-days of age. With the exception of the oil-treated group each rat received 2 μg of estrone daily for 3 days. The stippled area represents the "least significant difference" band for the estrone dose, and any group falling outside of this band is significantly different (5%) from the estrone-treated group. Each point represents the mean of 7 or 8 rats.

rone significantly inhibited the estrone-stimulated response at doses of 300 and 1000 μg. DCA inhibited this uterine growth at a dose of 300 μg, whereas, the higher doses (1000 and 3000 μg) did not have any effect. Although the actual uterine weights for certain of these inhibited groups fell below the level of the oil controls, none was significantly different. The two higher doses of estriol (100 and 1000 μg) significantly increased the response.

DISCUSSION

The attempt to resolve the differences between our conclusions and those of Velardo and his associates (8) was not entirely successful. We chose the 2 μg dose of estrone because of the considerable increment of growth they obtained with doses of this magnitude. The slight uterine growth produced in our study suggests a major difference in responsiveness between Sprague-Dawley and Charles River rats. Both testosterone propionate and estriol gave augmentation of the response rather than inhibition. Progesterone and low doses of DCA inhibited the response. High doses of DCA produce uterine growth (8) perhaps accounting for the absence of inhibition. Although these results show some differences in response between rats spayed at 100 days of age and the younger animals usually employed in this laboratory, this experiment does not show the patterns of inhibition of estrone reported by Velardo for estradiol-17β (8). Therefore, age of animals cannot entirely account for the discrepancies discussed previously (3).

The first two experiments presented in this paper appear to confirm the conclusions from previous studies, i.e., estrogen-induced growth of the uterus of young spayed rats may be inhibited by other steroids, but this inhibition is apparent only over a narrow range of doses. The estrone-progesterone study is particularly clear in this respect. The lower dose of progesterone produced significant inhibition of estrone at intermediate levels (0.3 to 3 μg); the higher and lower doses of estrone were not affected significantly by the progesterone. DCA did not have much effect upon estrone-induced uterine growth, although some inhibition of the response to 3 μg of estrone was suggested by the data. Previous studies are also instructive. Estriol at 3 μg inhibited estrone in this general range (1 and 3 μg only) whereas higher and lower estrone-doses were not affected, and 16-epiestriol inhibited only the response to the 1.0 μg dose (1). We have been able to inhibit uterine growth in rats with testosterone propionate only with the combination of 3 μg of estrone plus 100 μg of TP (3). Thus in every case in which we have found another steroid to inhibit the effects of estrone, the estrone was blocked at intermediate levels, particularly at 1 and 3 μg. The consistency of this pattern suggests that the significant inhibition reported above of 10 μg of estrone by 1000 μg of progesterone was spurious and strengthens the statistical argument for the estrone-DCA test. These data would seem to be in keeping with the concept of steroid buffering. How the effects of exogenous hormone are related to phenomena occurring

naturally in animals is obscure, but very low and very high hormone doses, producing little and maximal responses respectively, probably are unphysiological. Thus the doses that are most analogous to natural, endogenous hormone levels are presumably the intermediate doses that produce intermediate responses, and these are the levels that are subject to steroid inhibition. The hypothesis of steroid buffering (1, 3) assumes that these intermediate responses most closely represent normal uterine growth. The following components constitute the simplified buffering model:

1. When the primary estrogen (e.g., estrone) falls below titres necessary to maintain normal uterine growth, secondary estrogens (e.g., estriol) and androgens (e.g., testosterone) maintain the uterus at levels within normal limits.

2. When the primary estrogen begins to rise toward excessive levels, producing responses above functional limits, secondary estrogens, androgens, progestins (e.g., progesterone) and corticoids (e.g., desoxycorticosterone) inhibit the estrogen action, again maintaining normal uterine function.

It is interesting that four materials moderated the effect of rising estrogen titres, whereas only two augmented the response to low levels.

We are led to the conclusion that maintenance of normal uterine function is the result of concerted action of a variety of hormones augmenting or inhibiting one another in the manner of a chemically buffered system. In this conclusion we seem to be in general agreement with Velardo and his associates (8), differing only in particular details.

REFERENCES

1. Edgren, R. A. and D. W. Calhoun: *J. Endocrinol.* **20:** 325. 1960.
2. Edgren, R. A., D. W. Calhoun and T. W. Harris: *Anat. Rec.* **131:** 547. 1958.
3. Edgren, R. A., D. W. Calhoun and T. W. Harris: *Acta. endocrinol.* **34:** 213. 1960.
4. Edgren, R. A., R. L. Elton and D. W. Calhoun: *J. Reprod. & Fert.*, in press.
5. Hisaw, F. L., J. T. Velardo and C. M. Goolsby: *J. Clin. Endocrinol. & Metab.* **14:** 1134. 1954.
6. Velardo, J. T., F. L. Hisaw and A. T. Bever: *Endocrinology* **59:** 165. 1956.
7. Tukey, J. W.: *Biometrics* **5:** 232. 1949.
8. Velardo, J. T.: *Ann. N. Y. Acad. Sci.* **75:** 441. 1959.

ESTROGEN ANTAGONISMS: FURTHER REMARKS ON TEMPORAL ASPECTS OF STEROID BLOCK OF ESTROGEN-INDUCED VAGINAL SMEAR CHANGES IN MICE

RICHARD A. EDGREN[1]

Division of Biological Research, G. D. Searle and Co., Chicago, Illinois

ABSTRACT

In contrast to studies on rats, the vaginal effects of a single dose of estrone do not appear to be prolonged in mice by the simultaneous administration of progesterone. In fact, a 1000 μg dose of progesterone will antagonize 2 μg of estrone when both are given simultaneously in a single injection. Testosterone propionate and perhaps desoxycorticosterone acetate block estrone under the same conditions. When the same total doses of these materials are given in four daily injections, each of the blockers is an estrone antagonist. However, when the materials are given in two daily injections only testosterone antagonizes the estrone; desoxycorticosterone and progesterone are ineffective. No obvious or simple explanation of these effects presents itself.

THE temporal aspects of steroid blockage of estrone-induced vaginal effects in mice and rats have been the subject of a recent series of papers from this laboratory (mice: 1, 2, 3; rats: 4, 5, 6). Experiments with both rats and mice had shown that when estrone and blocker (progesterone, testosterone or desoxycorticosterone) were administered daily for four days, the estrogenic response was inhibited (2, 5). On the other hand, when estrogen and blocker were given for two days the estrogenic response was not affected by progesterone or desoxycorticosterone, in contrast to testosterone (1, 4). In the mice estrone and estriol appeared to have the same pattern of temporal relationships with the blockers (3). One of the most unexpected results that has emerged from this series of studies was the observation that a single dose of progesterone given to rats at the same time as estrone would prolong the effects of the estrogen (6). This study, which confirmed similar results of Maekawa (7) and Maekawa, Ito and Ikeda (8), also showed that estriol-induced changes were not prolonged by progesterone and that neither testosterone, desoxycorticosterone nor cortisone extended the duration of effect of estrone (6). The work to be reported here was initiated in the hope of quantifying estrogen-duration effects of progesterone administration in mice.

Received August 11, 1960.

[1] Present address: Research and Development Divisions, Wyeth Laboratories, Philadelphia, Pennsylvania.

MATERIALS AND METHODS

The methods employed here have been described in detail in earlier papers (1, 2, 3). To recapitulate: spayed mice were maintained in a colony and were selected weekly for testing. In the present series of experiments, each mouse received a single subcutaneous injection of 0.1 ml. of corn oil solution containing 2 μg of estrone. When a second steroid was to be administered it was mixed in solution with estrone and a single injection containing both materials in 0.1 ml. of corn oil was given.

Two separate experimental designs are to be reported. In the first, the duration of activity of the estrone, given alone, was compared with the duration of the estrone administered with various doses of progesterone. This was accomplished by examining vaginal smears taken from each mouse at 24-hour intervals starting 48 hours after injection of the steroids, and continuing until the estrone-treated animals had returned to the castrate condition.

In the second design, vaginal smears were taken from each mouse on the afternoon of the day after injection. Animals that had positive vaginal smears were returned to the colony, whereas a second smear was taken the next morning from those that had been negative. The temporal relationships between injection and smearing were the same in this experiment as those between smearing and the final injection of the series of injections described in earlier papers (1, 2, 3). One positive smear gave a particular mouse a positive score, and the results were pooled from the 32- and 48-hour smears. Blockers were administered at a total dose of 1000 μg.

The vaginal smears were considered positive if no leukocytes were present (9). Statistical significance was determined from the four-fold contingency test tables of Mainland and Murray (10).

RESULTS AND DISCUSSION

In these mice there was no evidence that progesterone prolonged the vaginal response to estrone (Table 1). At 48, 72 and 96 hours the 2 μg dose of estrone produced a response that remained essentially unchanged at a level of 33–50% positive. The addition of the lower three doses of progesterone (30, 100, 300 μg) did not significantly affect this estrogenic temporal pattern. The highest dose of progesterone (1000 μg) caused significant inhibition of the response on all three days. After 96 hours the smears showed a rapid return to the castrate condition. This absence of a proges-

TABLE 1. THE EFFECTS OF VARIOUS DOSES OF PROGESTERONE ON THE DURATION OF THE VAGINAL RESPONSE PRODUCED BY 2 μg OF ESTRONE

All compounds and mixtures given in a single injection

Dose (μg)		N	% positive smears				
Estrone	Progesterone		Time (hours after injection)				
			48	72	96	120	144
2	0	29	41	48	35	15	4
2	30	29	31	50	38	0	0
2	100	30	33	44	33	4	0
2	300	30	43	40	17	0	0
2	1000	30	7**	17*	4**	0	4

* Significantly different from estrone-treated group at 5% level.
** Significantly different from estrone-treated group at 1% level.

terone-induced increase in duration of the vaginal response to estrone is in marked contrast to our own rat studies (6). The most immediate conclusion that presents itself is that this represents a "species" difference between rats and mice, perhaps involving detoxification sites for estrogens. On the other hand, one must consider that 2 μg of estrone in the mice was a relatively more effective dose than the 10 μg given to the rats; the observed response duration in the mice was 72 hours whereas it was 24 hours in the rats. Perhaps this longer duration of estrone effect in the mice masked the prolongation that would have been obvious at lower dose levels. This does not seem entirely reasonable, since Maekawa (7) employed high estrone doses and administration schedules that gave high vaginal indices for 10 days or more.

The blockade of estrone by progesterone in this test was unexpected; earlier studies had suggested that the vaginal effects of estrone were blocked

TABLE 2. THE EFFECTS OF 1000 μG OF PROGESTERONE, DESOXYCORTICOSTERONE ACETATE OR TESTOSTERONE PROPIONATE ON VAGINAL SMEAR CHANGES PRODUCED BY 2 μG OF ESTRONE

These total doses of steroids were administered in a single injection 32 hours before the first smear. Mice that had failed to respond to the estrogen at the time of the first smear were reexamined the following morning.

Dose	N	Number positive	% positive
Estrone	31	24	78
Estrone + Progesterone	31	6	19**
Estrone + Desoxycorticosterone Acetate	29	16	55
Estrone + Testosterone Propionate	31	5	16**

** Significant from estrone-treated group at the 1% level.

by progesterone in long-term, but not in short-term tests (1, 2). Since the experimental protocol employed was different from that for the earlier studies, the second series of experiments was designed to obtain comparable data. 1000 μg of progesterone, or testosterone propionate (TP) blocked the vaginal effects of 2 μg of estrone (Table 2). The addition of desoxycorticosterone acetate failed to produce a statistically significant reduction in the response, although the actual reduction was of borderline significance, and was probably biologically meaningful.

A direct comparison of these data with those from the earlier papers is of particular interest (Table 3). In each experiment the estrone was given at a total dose of 2 μg and the blocker, whether it was testosterone propionate, progesterone or desoxycorticosterone acetate, was injected at a total does of 1000 μg simultaneously with the estrone. Estrone given on days 1, 2, 3 and 4 produced positive responses in about 90% of the mice. The addition of any of the possible antagonists resulted in a significant decrease in proportion of positive responses (2). When the estrone was given only on days 3 and 4 it appeared to have a somewhat lesser effect; in the experiments employing DCA and TP as antagonists only about ¾ of the rats showed

TABLE 3. COMPARISON OF THE EFFECTS OF 1000 μG DOSES OF DESOXYCORTICOSTERONE ACETATE, TESTOSTERONE PROPIONATE OR PROGESTERONE ON THE VAGINAL RESPONSE TO 2 μG OF ESTRONE

The tests differed in the proportion of material injected each day. Pairs of responses contained in each box are significantly different, at least at the 5% level. B = Blocker.

Day of injection				% positive response		
				Blocker		
Material				DCA	TP	Progesterone
1	2	3	4			
E	E	E	E	93 *	88 *	90 *
E+B	E+B	E+B	E+B	67	14	3
		E	E	76**	75 **	98**
		E+B	E+B	62	44	96
			E	78	78	78
			E+B	55	16	19

* Data from 2.
** Data from 1.

positive responses, whereas 98% was obtained in the progesterone experiment. Only testosterone produced a block of this response. Estrone on day 4 only gave 78% positive responses, which was significantly inhibited by progesterone and testosterone. In view of these data an early attempt at explaining temporal modalities in steroid block of estrogen-induced vaginal changes (2) seems naïve, but no simple explanation presents itself.

REFERENCES

1. EDGREN, R. A.: *Trans. Amer. Micros. Soc.* **79**: 33. 1960.
2. EDGREN, R. A.: *Acta Endocrinol.* **34**: 536. 1960.
3. EDGREN, R. A.: *Endocrinologia Japonica* **7**: 132. 1960.
4. EDGREN, R. A.: *Ann. N. Y. Acad. Sci.* **83**: 160. 1959.
5. EDGREN, R. A.: *Naturwissenschaften* **8**: 182. 1960.
6. EDGREN, R. A.: *Endocrinology* in press.
7. MAEKAWA, K.: *J. Faculty Sci., Univ. Tokyo* **7**: 455. 1955.
8. MAEKAWA, K., U. ITO AND Y. IKEDA: *Zool. Mag.* **68**: 29. 1959. Japanese with English Summary.
9. BIGGERS, J. D. AND P. J. CLARINGBOLD: *J. Endocrinol.* **11**: 277. 1954.
10. MAINLAND, D. AND I. M. MURRAY: *Science* **116**: 591. 1952.

GLYCOGEN IN THE UTERUS OF ALLOXAN-DIABETIC RATS[1]

RICHARD H. SWIGART, LAWRENCE G. GOLDBERG[2] AND WILLIAM B. ATKINSON

Department of Anatomy, University of Louisville School of Medicine, Louisville, Kentucky

ABSTRACT

The effect of estrogen on the concentration of glycogen was determined in tissue samples from the uterus, liver, diaphragm and urinary bladder of spayed, alloxan-diabetic rats.

Uterine glycogen was significantly increased in the alloxan-diabetic animals. Furthermore, uterine glycogen was significantly higher in the estrogen-treated alloxan-diabetic rats than it was after either alloxan treatment or estrogen treatment alone. These increases appeared to be related to the elevated blood sugar. The glycogen content of the urinary bladder was not altered.

SIGNIFICANT variations in the uterine glycogen content of the rat during the estrous cycle were first shown by Boettiger (1). This investigator also observed that when estrogen was injected into the ovariectomized rat it induced the deposition of glycogen in the uterus. Bo and Atkinson (2) demonstrated that the glycogen in the uterus of normal cyclic and of estrogen-treated ovariectomized rats is more heavily concentrated in the myometrium than in the endometrium as occurs in the uterus of primates (3). Kostyo (4) studied the effect of several other steroids on uterine glycogen in the ovariectomized rat. He found that testosterone and desoxycorticosterone induced the deposition of uterine glycogen. Cortisone, cortisol, 11-desoxycortisol and pregnenolene, on the other hand, failed to show any effect.

A number of experimental procedures have been performed to study their effects upon the deposition of uterine glycogen in the estrogen-treated ovariectomized rat. The procedures which have failed to alter the quantity of glycogen deposited are hypophysectomy (5), adrenalectomy (5, 6), fasting (2, 6), hypophysectomy and fasting concurrently (7), the subcutaneous injection of adrenalin (6, 8), and the subcutaneous injection of cortisone and cortisol (9). On the other hand, the subcutaneous injection of relaxin increased the quantity of glycogen deposited (9), whereas intraperitoneal injection of posterior pituitary extract (8), of epinephrine (8), and of corti-

Received August 18, 1960.

[1] This study was supported in part by Grants No. C-4510(C1) and No. H-4734(C1) from the National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

[2] Summer Research Scholar on a grant from the Kentucky Heart Association.

sone and epinephrine concurrently (4), decreased the quantity of glycogen deposited in the estrogen-treated ovariectomized animal.

The present study was undertaken to determine whether alloxan diabetes, with its accompanying elevated blood sugar, has any effect upon the uterine glycogen content in the untreated and estrogen-treated ovariectomized rat. Furthermore, the glycogen content of the urinary bladder was determined. This afforded an opportunity to compare the values obtained for the uterus with those of an organ outside of the female reproductive system possessing a comparable amount of smooth muscle. Finally, samples of the liver and diaphragm were analyzed to substantiate further the state of diabetes indicated by the high blood sugar levels occurring in nonstarved animals following treatment with alloxan.

METHODS AND MATERIALS

Female albino rats of the Wistar strain, 55–60 days of age and weighing between 150 and 175 grams, were bilaterally ovariectomized. Following a 10-day recovery period the animals were randomized into four groups as follows: (1) twelve animals served as controls and received no further treatment before being sacrificed; (2) twelve animals were injected subcutaneously with 10 μg of estradiol benzoate and sacrificed 72 hours later; (3) ten animals were injected subcutaneously with 175 mg. of alloxan/kg. body weight and sacrificed 7 days later; (4) ten animals were injected subcutaneously with 175 mg. of alloxan/kg. body weight and 5 days later were injected subcutaneously with 10 μg of estradiol benzoate and sacrificed after 72 hours. The blood glucose was determined in all alloxan-treated animals on the third day after alloxan injection. Also, the levels of blood glucose were determined in all animals on the day they were sacrificed. The blood was obtained by clipping the tail of each rat while under light ether anesthesia. The blood was deproteinized according to the micro-procedure of Somogyi (10) and its glucose content determined by the method of Nelson (11). After blood was taken on the final day, sufficient time (usually 4 to 5 hours) was allowed to elapse for the animals to recover from the ether anesthesia to which they had been exposed. The animals were then killed by a blow to the occiput, followed by decapitation. Tissue samples from the right and left horns of the uterus, liver, diaphragm and the urinary bladder were quickly removed and frozen in wells drilled in a 3/4 inch aluminum plate which was packed in powdered dry ice. The samples were weighed in the frozen state and then lyophilized. The dried tissues were digested with 30% KOH. The glycogen was precipitated and hydrolyzed according to the method of Walaas and Walaas (12). The final glucose concentrations of the hydrolysates were determined by the Nelson method.

RESULTS AND DISCUSSION

The average values for blood sugar, uterine glycogen and urinary bladder glycogen for the four experimental groups are shown in Figure 1. Glycogen concentrations were determined in both horns of the uterus in each animal. Since the values from the two horns were not significantly different, these data have been combined. In the untreated control animals the blood glucose and uterine glycogen values compare favorably with those obtained by previous authors (8, 13). Values for urinary bladder glycogen, however, have not been found in the literature. From Figure 1 it may be seen that in

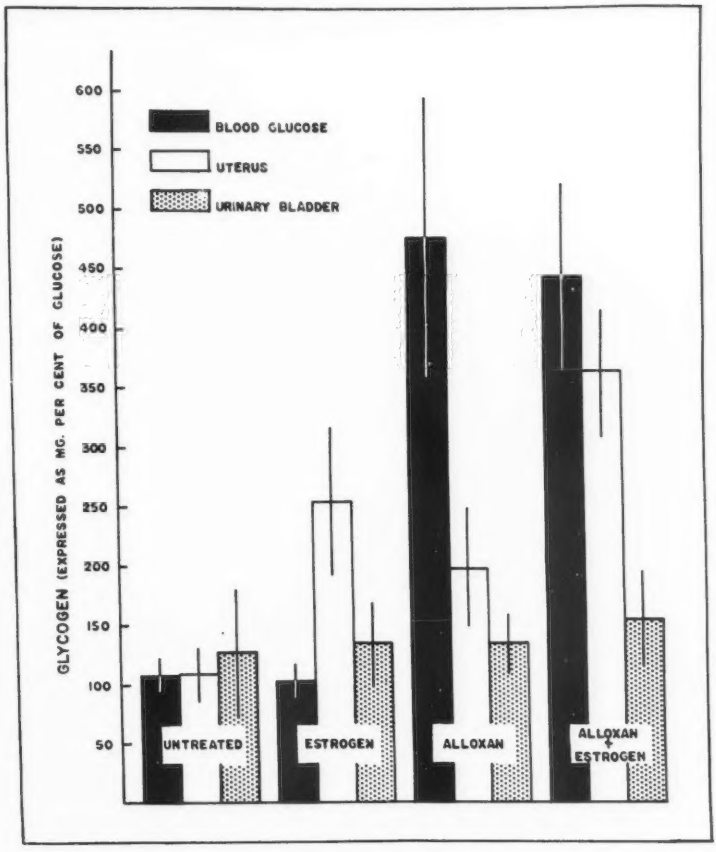

FIG. 1. Blood glucose, uterine glycogen and urinary bladder glycogen in control, estrogen-treated, alloxan-diabetic, and estrogen-treated alloxan-diabetic rats following ovariectomy. The vertical lines at the tops of the bars indicate the standard deviation.

the estrogen-treated animals the blood sugar and urinary bladder glycogen were not significantly different from the levels present in the untreated rats. The increase in uterine glycogen reported by previous investigators (1, 2) was confirmed. In the alloxan-diabetic rats the blood glucose was quite high. The glycogen in the urinary bladder remained unchanged while the glycogen content of the uterus increased significantly. Finally, in the estrogen-treated alloxan-diabetic rats a high blood glucose was also observed and the urinary bladder glycogen was still unchanged from the control value. However, the uterine glycogen content was significantly higher than in either the estrogen-treated or alloxan-diabetic animals. The probabilities of the differences found between the uterine glycogen contents of the untreated and estrogen-treated animals, of the untreated and alloxan-

diabetic animals, of the estrogen-treated and estrogen-treated alloxan-diabetic animals, and of the alloxan-diabetic and estrogen-treated alloxan-diabetic animals being due to chance were calculated and the P values were less than .001 in all instances.

Liver and skeletal muscle normally contain relatively high concentrations of glycogen, but the amount present is depleted in diabetes when blood insulin levels fall. It is generally agreed that insulin is necessary for the uptake of glucose by hepatic and skeletal muscle cells, though the mode of action is controversial. On the other hand, the present experiments demonstrated that the glycogen content of the smooth muscle of the uterus was increased in alloxan-diabetic animals in which the blood glucose was elevated and the liver and skeletal muscle glycogen content had fallen. It would seem from these observations that insulin (at least in concentrations necessary to maintain liver glycogen) is not necessary for the uptake of glucose by the smooth muscle of the uterus, but rather that the uterine glycogen content is related directly to the blood sugar level. This is further substantiated by the finding that the uterine glycogen content is significantly higher in estrogen-treated alloxan-diabetic rats than in estrogen-treated animals without diabetes. It is not meant to imply that insulin has no effect on the uptake of blood glucose by the myometrium. This, in fact, has not been tested in the present experiment. It can only be concluded that the glycogen content of the myometrium is related in some way to the concentration of glucose available in the blood.

REFERENCES

1. Boettiger, Edward G.: *J. Cell. and Comp. Physiol.* **27**: 9. 1946.
2. Bo, Walter J. and William B. Atkinson: *Anat. Rec.* **133**: 91. 1952.
3. van Dyke, H. B. and G. Chen: *Am. J. Anat.* **58**: 473. 1936.
4. Kostyo, Jack L.: *Proc. Soc. Exper. Biol. & Med.* **97**: 405. 1958.
5. Atkinson, William B. and Roger C. Crafts: *Anat. Rec.* **113**: 101. 1952.
6. Walaas, Otto: *Acta. Endocrin.* **10**: 175. 1952.
7. Bo, Walter J. and William B. Atkinson: *Proc. Soc. Exper. Biol. & Med.* **83**: 405 1953.
8. Kostyo, Jack L. and Samuel L. Leonard: *Endocrinology* **56**: 616. 1955.
9. Steinetz, B. G., V. L. Beach, R. P. Blye and R. L. Kroc: *Endocrinology* **61**: 287. 1957.
10. Somogyi, M.: *J. Biol. Chem.* **86**: 655. 1930.
11. Nelson, N. J.: *J. Biol. Chem.* **153**: 375. 1944.
12. Walaas, O. and E. Walaas: *J. Biol. Chem.* **187**: 769. 1950.
13. Guest, M. M.: *J. Nutrition* **22**: 205. 1941.

CHARACTERIZATION OF THE ANTERIOR PITUITARY FACTOR WHICH ANTAGONIZES GONADOTROPHINS[1]

MARION C. WOODS AND MIRIAM E. SIMPSON

The University of California, Institute of Experimental Biology, Berkeley, and the Department of Anatomy, San Francisco, California

ABSTRACT

The anterior pituitary contains a substance (or substances), tentatively called the "antagonist," which, on intraperitoneal administration, reduces the ovarian response to simultaneously administered gonadotrophins. During purification of anterior pituitary hormones this factor has most consistently been associated with the gonadotrophic fractions and in particular with the interstitial cell stimulating hormone (ICSH). Evidence is here presented which indicates that antagonism is not, however, an intrinsic property of sheep ICSH but is due to an undetermined substance which follows closely during purification and is still present in preparations of ICSH with a minimal effective dose of 1 μg. The antagonistic properties characteristic of partially purified follicle stimulating hormone (FSH) have been shown to disappear on continued purification.

IT HAS been demonstrated that certain pituitary extracts can prevent or depress the ovarian response of immature rats to various gonadotrophic stimuli, including the gonadotrophic stimulus of the animal's own pituitary or of extracts or implants of anterior lobe (1–5), human chorionic gonadotrophin (6–9), pregnant mare serum (7, 9–13), human menopausal hormone (12) and (partially purified) pituitary gonadotrophins (4, 9–11, 14–16). This phenomenon of antagonism has been characterized by less ovarian development than that given by the gonadotrophic stimulus alone and has frequently been accompanied by increased luteinization and follicular atresia, and by less evidence of estrogen secretion, as indicated by reduced uterine and vaginal responses. It has been noted that the route of injection (intraperitoneal or intravenous) was of great importance in demonstration of the phenomenon of antagonism (7, 9, 14). It is improbable that antagonism is to be considered due to antihormone formation, since the duration of the test is brief (72 to 120 hours).

The antagonistic principle has been reported as present with several of the partially purified anterior pituitary hormones: growth hormone (3, 15), interstitial cell stimulating hormone (ICSH) or luteinizing hormone (LH) (8, 11, 13, 16, 17), and follicle stimulating hormone (FSH) (11, 14). No non-

Received September 1, 1960.

[1] Aided by a grant from the Population Council, New York, N. Y., and by United States Public Health Service Grant A-800.

pituitary gonadotrophin has been found to have antigonadotrophic properties (8, 10), and no non-pituitary tissue source has been reported. That antagonism is not due to the growth hormone in growth promoting extracts was first demonstrated by Leonard (4) and, though he found the antagonistic factor associated with LH fractions, he concluded that LH was probably not the antagonist since LH from horse pituitary was not effective as an antagonist (8). Fevold and Fiske (18), in considering the reduced ovarian weights associated with the luteinization following estrogen injection, concluded that the phenomenon of antagonism is attributable either to LH itself or to secretion of an antagonistic factor by the pituitary under the influence of estrogen. Fraenkel-Conrat *et al.* (11) found that the most highly purified FSH and ICSH preparations then available were both capable of exerting antagonism. The phenomenon has either not been demonstrated or has not been investigated in adult female rats (9). Antagonism is readily demonstrable in the absence of the pituitary (2, 9–12, 14, 17). Fraenkel-Conrat *et al.* (11, 12) could not, however, demonstrate antagonism by their most purified sheep ICSH preparations in hypophysectomized female rats.

Antagonism in the male has not been effectively demonstrated. The few observations which could be interpreted as showing antagonism must be considered in relation to the injurious effect exerted by posterior lobe principle on the testis (2, 19, 20, 21).

The purpose of the present investigation was to analyze the phenomenon of antagonism in normal and hypophysectomized immature female rats, using FSH and ICSH of greater purity than had previously been utilized in such studies.

METHODS AND MATERIALS

Bioassay Procedure: Normal immature female rats of the Long-Evans strain, weighing 50 to 65 grams and 25 days of age, were injected subcutaneously, once daily for four days, with the primary gonadotrophic stimulus. Simultaneous daily injections of the substance being tested for antagonism were given intraperitoneally. A minimum of six rats per experimental group was used and all critical assays were redetermined repeatedly. The hormonal preparations were administered as solutions in 0.9% NaCl. Autopsy was performed 96 hours after the first of four daily injections. The primary gonadotrophic stimulus utilized most frequently in normal immature recipients was human chorionic gonadotrophin (HCG). A *uterine unit* of antagonism was determined to be that dose, in a series of graded doses, which produced 50% inhibition of the uterine weight caused by the gonadotrophic stimulus of 4 I.U. of HCG given alone. An *ovarian unit* of antagonism was the dose which caused 50% inhibition of the ovarian weight produced by 20 I.U. of HCG alone. Determination of the dosage which produced 50% ovarian and uterine inhibition was made by interpolation from plots of log-dosage against per cent reduction of organ weight, such curves being based on three or more points. An endpoint of 50% reduction in weight was adopted as a more rigorous test for antagonism than the 25% reduction adopted in previous studies (10, 11). HCG, rather than equine serum gonadotrophin (PMS), was adopted as the primary stimulus for use in normal animals, since the organ weights resulting from its use were less variable. (The

higher unitage of HCG was chosen for determination of the ovarian unit since ovarian weight increases in animals receiving 4 I.U. HCG were too small and erratic to be a basis for calculation. On the other hand, the uteri of animals receiving 20 I.U. HCG were usually so greatly stimulated that the antagonism phenomenon was obscured.)

Hypophysectomized female rats. In order to analyze the phenomenon of antagonism in the absence of the pituitary, female rats, 26 to 28 days of age, weighing 55 to 80 grams, were hypophysectomized and observed for completeness of hypophysectomy during seven days before injections were started. At autopsy the completeness of the hypophysectomy was confirmed by the usual careful examination of the pituitary site and, where indicated, by histological examination of pituitary target organs. The injection schedule already described for normal recipients was followed without variation. Histological confirmation of ovarian changes noted at autopsy was made in all cases. The primary gonadotrophic stimulus was routinely PMS.

Standardization of FSH and ICSH. FSH and ICSH potencies were determined in immature hypophysectomized female rats by the method of Evans *et al.* (10), as described again recently by Woods and Simpson (22). The rat unit (R.U.) of FSH, as used here, is the minimal effective dose (MED) for follicular development, and that for ICSH is the MED for interstitial repair (total dose in 3 days).

Materials for injection. The human chorionic gonadotrophin (HCG; Antophysin, Winthrop) used in these studies had a potency of 20 I.U. per mg. The equine pregnancy serum gonadotrophin (PMS; Gonadogen, Upjohn) had a potency of 1000 I.U. per cc. The human menopausal urinary gonadotrophin (HMG-J_5, S. Johnsen, Statens Serum-institut, Copenhagen) had an MED for follicular development in the hypophysectomized rat of 75 μg.

The normal (N) and gonadectomized ($\overline{G}$) adult male rat anterior pituitaries used in these studies were injected as homogenates in 0.9% NaCl. Fresh preparations were made for each 4-day experiment, the emulsion being stored at 5° C during this period. Gonadectomy was performed at 50 to 80 days of age. The blood plasma of these animals, as well as the pituitaries, was collected 4 to 6 weeks postoperatively. The plasma from gonadectomized donors was precipitated in 80% ethanol at 0° C; the precipitate was then extracted with water, dialyzed and lyophilized. A dose of this preparation equivalent to 10 cc. plasma gave minimal follicular stimulation in hypophysectomized recipients. The gonadectomized adult rat anterior lobes were also extracted with 50% ethanol and treated by the procedure used in preparing extracts of sheep pituitaries (22). The MED for FSH in the ethanol extracts of castrate rat pituitaries was 30 μg; the MED for ICSH, 5 μg; the yield was 0.37 mg. per rat.

The sheep pituitary FSH and ICSH preparations, examined for content of antagonist, were fractionated as described in the addendum to this report. For purposes of comparison, the following preparations from sheep pituitaries were assayed: sheep LH XIII-25-2 from S. Ellis, Department of Biochemistry, Emory University; NIH-LH-1 and NIH-FSH-1, distributed by the National Institutes of Health, also prepared according to the method of Ellis (23); Armour standards LH 227-80 and FSH 264-151-X, provided by the Biochemistry Research Department, Armour Pharmaceutical Co. The lactogenic hormone preparation from sheep pituitaries (NIH-SP-1), estimated to contain 15 I.U. per mg., was distributed by the Endocrine Study Section of the NIH. Sheep growth hormone (G-43) was prepared by the method of Ellis *et al.* (24) and was shown by standardization in immature hypophysectomized female rats by the tibia test (25, 26) to have a potency of 40 μg. Sheep thyrotrophic hormone (VII-N-36) was prepared for this study by the method of Fels (27), and was determined histologically (28) to have an MED of 5 μg for thyroid repair in the hypophysectomized female rat. The sheep adrenocorticotrophic preparation LLR-82, prepared by L. L. Rosenberg, was an oxycellulose eluate containing 2 U.S.P. units per mg.; preparation LLR-110-D-1 was

further purified by chromatography on IRC-50 and contained 100 U.S.P. units per mg. (29).

A posterior pituitary hormone preparation was obtained by homogenization of dissected, lyophilized monkey posterior lobes. Isolated vasopressin and oxytocin were obtained from Parke, Davis and Co. (Pitressin, 20 pressor units per cc., and Pitocin, 10 units per cc.).

Proteinase I was prepared according to Ellis (23) and was standardized for proteolytic activity according to Adams and Smith (30).

RESULTS

Non-Gonadotrophic Hormones. Antagonism was not exhibited by any of the non-gonadotrophic pituitary preparations in the dose range tested (Table 1). This confirms previous findings (4, 8, 9), and is also in accord with the observation that the phenomenon of antagonism can be demonstrated in thyroidectomized and adrenalectomized rats (9).

TABLE 1. PITUITARY PRINCIPLES TESTED AND FOUND LACKING ANTAGONISTIC PROPERTIES
(Assayed in normal immature female rats against 4 I.U. HCG, SC)

Preparation	Source	Unitage	Dose range tested
Growth hormone	Sheep, G-28	R.U. 40 μg	3–15 R.U.
Adrenocorticotrophic hormone	Sheep, LLR-82	2 U.S.P. U./mg.	100–500 μg
	LLR-110-D-1	100 U.S.P. U./mg.	10–100 μg
Thyrotrophic hormone	Sheep, VII-N-36	R.U. 5 μg	3–30 R.U.
Lactogenic hormone	NIH-SP-1	15 I.U./mg.	0.5–10 I.U.
Proteinase I	Sheep, (23)	40 Adams-Smith U./mg.	100–1000 μg
Posterior pituitary:			
1. Whole lobe	Monkey		50–500 μg
2. Vasopressin	Pitressin, P.D.&Co.		2–8 pressor U.
3. Oxytocin	Pitocin, P.D.&Co.		2–8 I.U.

FSH. Due to the intrinsic properties of FSH preparations, it is difficult to assay accurately the presence of the antagonistic factor. Antagonism could be demonstrated, however, in preparations of sheep FSH under limited circumstances (Table 2). Cruder preparations of sheep FSH, those, for example, with MED 50 μg, had the capacity to antagonize the gonadotrophic action of HCG in normal immature rats, though only when administered in low doses (1 to 2 R.U.).[2] Purer preparations, with MED 20 μg or less, displayed no antagonism against gonadotrophic agents (HCG, PMS, HMG) or against FSH itself in either normal or hypophysectomized rats at any level examined (0.5 to 200 R.U.). Preparations of FSH with MED of 20 to 5 μg retained their effectiveness in normal as well as in hypophysectomized rats, against rat pituitary gonadotrophin (from normal [N] and gonadectomized [G] rats) provided the doses of FSH, as well as

[2] This statement is equally true of FSH preparations made elsewhere; NIH-FSH-S1 (MED 50 μg) having a uterine unit for antagonism in 1 to 2 R.U. FSH, was decreasingly effective at higher dosages. A more purified preparation from S. Ellis (XIII-13-3, MED 15 μg) was ineffective at 1–3 R.U. FSH.

rat pituitary, were carefully selected.[3] Preparations of FSH with MED less than 5 μg did not display the phenomenon of antagonism even under these conditions.

The greater effectiveness of FSH preparations in stimulation of follicles when administered subcutaneously rather than intraperitoneally has been interpreted as evidence for antagonism by FSH (10). With continued purification of FSH to MED less than 50 μg, this difference in the route of injection has also disappeared (22).

TABLE 2. ANTAGONISM OF VARIOUS PRIMARY GONADOTROPHIC STIMULI SHOWN BY FSH AND ICSH PREPARATIONS AT PROGRESSIVE STAGES OF PURIFICATION

Primary gonadotrophic stimulus* SC	Pituitary fraction assayed as antagonist, IP									
	FSH†						ICSH†			
	MED, μg	N		$\overline{H}$			MED, μg	N		$\overline{H}$	
Homogenate, rat or sheep pit. HCG	500–25	+ +	(+)‡	+	(+)		500–50	+ +		+	
Homogenate, N or $\overline{G}$ ♂ rat pit.	20– 5	+ to ±		+ to ±			35– 5	+		+ to ±	
40% EtOH extr., $\overline{G}$ ♂ rat pit.		+ to ±		−						−	
Plasma, $\overline{G}$ rat				−						+	
Sheep FSH (MED 10–20 μg)		−	(+)	−	(+ to ±)			−	(−)	−	(−)
PMS		−	(+)	−	(+)			+	(+)	+	(−)
HCG		−						+	(+)		
HMG			(+)	−					(+)	−	
40% EtOH extr., $\overline{G}$ ♂ rat pit.	5–1.7	−		−			5– 1			+ to ±	
FSH (MED 4 μg)										−	
PMS			(+)					+		−	
HCG								+			
HMG		−		−				−		−	

* Minimum of six 25 day old female rats per group; 4 days injection; dose adjusted to produce 35 to 50 mg. ovarian wt.

† FSH and ICSH are tested over a dose range of 0.5 to 200 R.U.

‡ Plus or minus in parentheses refers to studies of Fraenkel-Conrat *et al.* (11) (31), except rat pituitary homogenates, which refer to Talbert *et al.* (14).

ICSH. All types of ICSH preparations, even those most highly purified, having MED 1 μg, were found to act as antagonists, reducing the effectiveness in normal rats of simultaneously injected HCG (Fig. 1, Table 2). The results of testing 41 ICSH preparations as antagonists against HCG in immature normal rats are presented in Table 3. As purity was improved, the ability to antagonize became more variable, and much higher unitages were frequently required for 50% reduction of ovarian or uterine weights. Figures 2 and 3 present these data as scatter diagrams. This can be interpreted as indicating that the antagonistic factor is not to be identified

[3] The use of pituitary homogenates permitted demonstration of either augmentation or antagonism, depending on the dosage; whereas one-fourth gland, given subcutaneously with either FSH or ICSH intraperitoneally, resulted in augmentation, one-half gland or more gave antagonism under the same conditions.

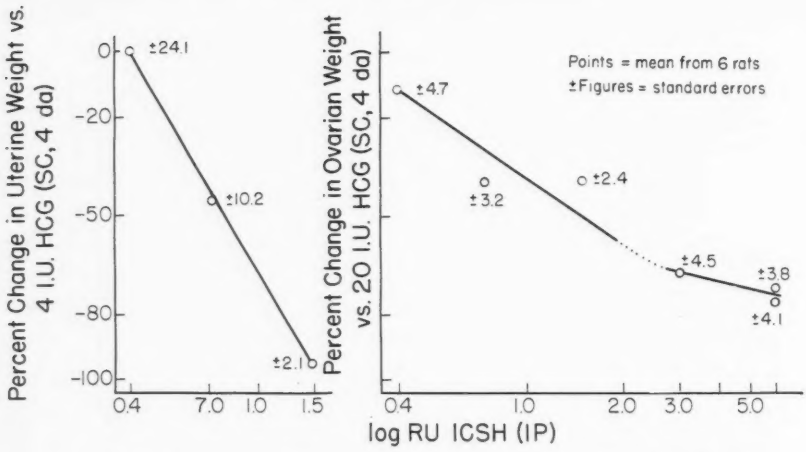

FIG. 1. Antagonism in normal 25 day-old Long-Evans rats to HCG by sheep ICSH (I-10Y, MED 20 μg).

with ICSH.[4] It should be noted here that ICSH of potency 1 μg was still not pure, as evidenced by lack of chromatographic homogeneity and by the presence of traces of other contaminants (TSH, Proteinase I).

Although certain treatments during the purification of ICSH may prove to be responsible for the decrease in antagonism, no single purification procedure has been discovered which will consistently and completely eliminate antagonism as a property of ICSH preparations. Ammonium sulfate fractionation produced only partial and inconsistent elimination of this property. Although ion-exchange chromatography on DEAE-cellulose at very low ionic strength appeared promising, the results were inconclusive since fractionation under these conditions was difficult to control and to reproduce. Precipitation of ICSH of MED 35 μg and 3 μg with 10% trichloracetic acid at 3° C, quickly followed by dialysis and lyophilization, significantly reduced antagonism without altering ICSH potency (Fig. 4). (Similar treatment of ICSH of MED 1 μg resulted in a loss of over 80% of the ICSH potency.)

The effectiveness of ICSH preparations as antagonists in hypophysectomized rats was lost earlier during purification procedures than that in intact rats. Preparations of ICSH with MED of 5 μg or less were ineffective

[4] Correlation coefficients for antagonism unitage with ICSH unitage were +0.7 for the ovarian unit, and +0.8 for the uterine unit (+0.9 for the uterine unit of ICSH preparations from aqueous extracts, +0.7 for preparations from ethanol extracts).

$$r = \text{correlation coefficient} = \frac{N\Sigma XY - (\Sigma X)(\Sigma Y)}{\sqrt{[N\Sigma X^2 - (\Sigma X)^2][N\Sigma Y^2 - (\Sigma Y)^2]}}$$

A positive correlation is indicated by r = +0.8 to 1.0 (32).

TABLE 3. ANTAGONISM OF THE GONADOTROPHIC ACTION OF HCG IN NORMAL IMMATURE FEMALE RATS SHOWN BY VARIOUS SHEEP ICSH PREPARATIONS

Part A. Fractions derived from aqueous extracts

Preparations of ICSH assayed						Antagonism unit expressed as a multiple of MED for ICSH	
No.	MED ICSH, μg	Yield mg./kg. wet glands	Derived from				
			Fraction	MED ICSH, μg	Procedure	Uterine unit	Ovarian unit
1	100	900	Wet glands	5000	Extr. pH 9; insol. 1.2M $(NH_4)_2SO_4$ pH 7.5	8.0	13
2	35	1280			Sol. 1.2M $(NH_4)_2SO_4$ pH 7.5; insol. 2.0M $(NH_4)_2SO_4$; sol. 1.2M $(NH_4)_2SO_4$ pH 4.7	3.5	9.5
3	35	260			Insol. 1.2M $(NH_4)_2SO_4$ pH 4.7	1.4	3.5
4	35	1025	(No. 2)	35	Insol. 10% TCA, 3° C	8.0	30
5	50	980	Extr. pH 9;	—	Insol. 1.2M $(NH_4)_2SO_4$ pH 7.3	6.0	15.5
6	40	370	2.0M $(NH_4)_2SO_4$ pH 5 insol.		Sol. 1.2M $(NH_4)_2SO_4$ pH 7.3, but insol. 1.2M $(NH_4)_2SO_4$ pH 4.7	3.0	15.5
7	15	1400			Sol. 1.2M $(NH_4)_2SO_4$ pH 4.7 and 7.3	7.5	12
8	12.5	1600	Extr. pH 9; 2.0M $(NH_4)_2SO_4$ pH 5 insol.	—	Sol. 0.1M $(NH_4)_2SO_4$ pH 7.3; insol. 1.2–1.8M $(NH_4)_2SO_4$ pH 5	4.0	7.2
9	10	1300	Extr. pH 9; 2.0M $(NH_4)_2SO_4$ pH 5 insol.	—	Sol. 0.1M $(NH_4)_2SO_4$ pH 7.3; insol. 2.0M $(NH_4)_2SO_4$ and sol. pH 4.1 (HPO_3); IRC-50 (ref. 23)	4.0	16.5
10	7.5		Extr. pH 7.5		Insol. 2.2M $(NH_4)_2SO_4$ pH 7.5; sol. 1.25M $(NH_4)_2SO_4$ pH 5.5 and 4.0; sol. pH 4.1 (HPO_3) (S. Ellis)	4.0	—
11	5	300			IRC-50 (NIH-LH-S1)	2.0	25
12	5	250	Extr. pH 9; Insol. 1.2–1.8M $(NH_4)_2SO_4$, pH 5	15	DEAE-C, elution 0.005M PO_4 pH 7.0	2.0	—
13	5	310	Extr. pH 9; Insol. 1.2–1.8M $(NH_4)_2SO_4$, pH 5	5	DEAE-C, elution 0.005M PO_4 pH 7; sol. pH 4.1 (HPO_3)	3.5	18
14	5	570	Extr. pH 9; Insol. 1.2–1.8M $(NH_4)_2SO_4$,pH5	12.5	Sol. pH 4.1 (HPO_3); DEAE in H_2O; elution 0.005M PO_4 pH 7	5.0	170
15	3	230	(No. 7)	15	DEAE-C in H_2O (not retained)	4.0	95
16	3	110	Pooled from DEAE-C	3–5	DEAE-C in H_2O pH 6.2; elution 0.005M PO_4 pH 7.0	4.0	25
17	3	75	(No. 16)	3	Insol. 10% TCA 3° C	10.0	105
18	2.0	35	(No. 15)	3	IRC-50: pH 8-borate-PO_4; elution 1M NaCl (ref. 23)	4.0	55
19	2.0	35	(No. 16)	3	IRC-50: pH 6.0 0.2M PO_4; elution pH 6.85 (ref. 42)	5.0	85
20	1.0	30	(No. 16)	3	IRC-50: pH 6.0 0.2M PO_4; elution pH 6.85 (ref. 42)	8.0	85
21	1.7	14	(No. 20)	1.0	IRC-50: 0.2M PO_4 pH 6.0 (not retained)	7.5	120
22	1.3	4.5			Elution 0.2M PO_4 pH 6.9	4.5	35

as antagonists in hypophysectomized rats though assayed over a range of 0.5 to 200 R.U. (Table 2). This finding is consistent with the observations of Fraenkel-Conrat *et al.* (11). Table 4 illustrates the change in reaction of hypophysectomized recipients as purity of ICSH is increased. Significant suppression of ovarian and uterine development was demonstrated with the preparation with MED 35 μg when assayed against appropriate levels of

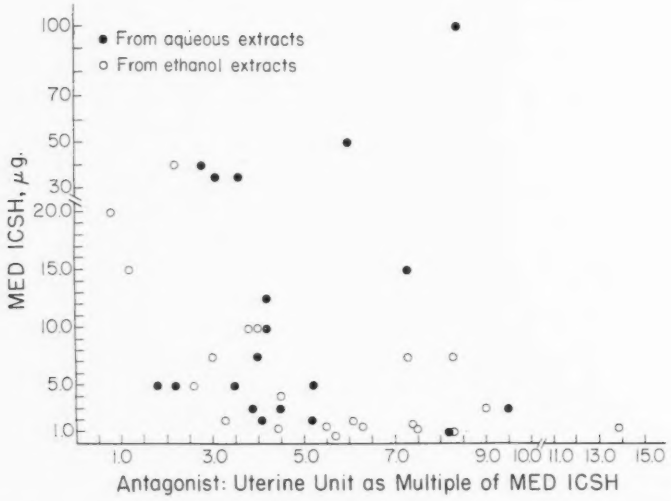

FIG. 2. Antagonism by sheep ICSH (IP) in normal immature rats. Uterine unit = 50% inhibition of uterine weight. Produced by 4 I.U. HCG (4d, SC) alone.

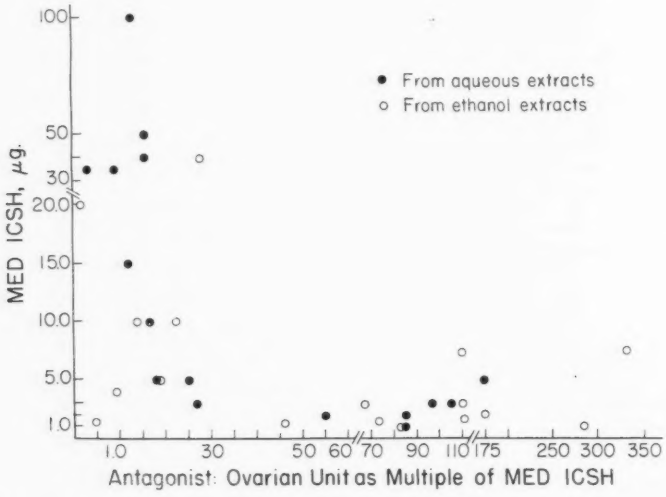

FIG. 3. Antagonism by sheep ICSH (IP) in normal immature rats. Ovarian unit = 50% inhibition of ovarian weight. Produced by 20 I.U. HCG (4d, SC) alone.

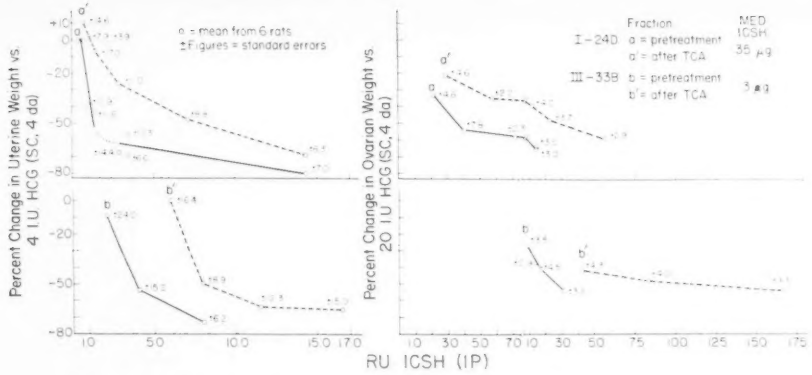
o = mean from 6 rats
± Figures = standard errors
Fraction
MED ICSH
I–240 a = pretreatment
a' = after TCA
35 μg
III–338 b = pretreatment
b' = after TCA
5 μg
Percent Change in Uterine Weight vs. 4 I.U. HCG (SC, 4 da)
Percent Change in Ovarian Weight vs. 20 I.U. HCG (SC, 4 da)
RU ICSH (IP)

FIG. 4. Antagonism in normal immature rats to HCG by sheep ICSH after treatment with 10% TCA, +3° C.

TABLE 3. ANTAGONISM OF THE GONADOTROPHIC ACTION OF HCG IN NORMAL IMMATURE FEMALE RATS SHOWN BY VARIOUS SHEEP ICSH PREPARATIONS

Part B. Fractions derived from 40% EtOH extracts

Preparations of ICSH assayed						Antagonism unit expressed as a multiple of MED for ICSH	
No.	MED ICSH, μg	Yield, mg./kg. wet glands	Derived from: Fraction	MED ICSH, μg	Procedure	Uterine unit	Ovarian unit
23	40	35	Wet glands	5000	Extr. 40% EtOH 3° C; 1.4–1.8M $(NH_4)_2SO_4$	2.0	28
24	20	95	40% EtOH extr. 25° C; yield 8 gm.	200	1.4–1.9M $(NH_4)_2SO_4$	1.0	2
25	10	35	40% EtOH extr. 25° C; yield 8 gm.	200	1.2–2.0M $(NH_4)_2SO_4$; dial. insol.	4.0	25
26	15	40			Dial. sol.	1.0	> 35
27	10	95	40% EtOH extr. 25° C; yield 8 gm.	75	1.4–1.8M $(NH_4)_2SO_4$	4.0	15
28	5	85	40% EtOH extr. 25° C; yield 8 gm.	150	1.4–1.8M $(NH_4)_2SO_4$	2.5	20
29	3	40	50% EtOH extr. 25° C; yield 4 gm.	30	1.4–1.8M $(NH_4)_2SO_4$	6.0	35
30	1.3	3.3	(No. 25, 26)	10–15	DEAE-C pH 7 H_2O; (not retained)	13.0	45
31	2.0	3.5			Elution 0.005M PO_4 pH 7	3.0	175
32	4	3.7			Elution 0.015M PO_4 pH 7	4.5	9
33	1.5	5.2	1.2–2M $(NH_4)_2SO_4$	5–6	DEAE-C; elution 0.005M PO_4 pH 7.2	5.5	45
34	1.5	5.2	(No. 33)	1.5	6M NH_4OH 25° C 24 hr.; dial.	6.5	75
35	7.5	5.2	(No. 33)	1.5	6M urea pH 8 40° C 24 hr.; dial.	8.5	120
36	0.75	20	50% EtOH; 1.4–1.8M $(NH_4)_2SO_4$	5–8	DEAE-C; elution 0.005M PO_4 pH 7	5.5	280
37	3	7	(No. 36)	0.75	IRC-50: 0.2M PO_4 pH 6.0 (not retained)	9.0	35
38	7.5	3			Elution 0.2M PO_4 pH 6.9	7.5	330
39	1.3	2.3	50% EtOH; 1.4–1.8M $(NH_4)_2SO_4$	3.0	DEAE-C; elution 0.005M NH_4OAc pH 9.3	7.5	20
40	1.0	4.0			DEAE-C; elution 0.015M NH_4OAc pH 9.3	8.5	85
41	>15	1.0	(No. 39)	1.3	10% TCA 3° C; dial.	17	170

TABLE 4. ANTAGONISM OF GONADOTROPHIC ACTION OF PMS IN HYPOPHYSECTOMIZED RATS BY ICSH PREPARATIONS OF WIDELY DIFFERENT POTENCY

Total dose			No. of rats	Ovaries				Uterus		
PMS, I.U., SQ	ICSH			Weight, mg.		Follicles	Corpora lutea	Weight, mg.		Status
	MED, μg	R.U., IP		Av.	(Range)			Av.	(Range)	
20	35	—	3	33	(27– 39)	small to medium thecal hypertr.	+	63	(40–104)	medium
		5	2	18	(16– 20)	few, small to medium atretic	+ (fewer)	34	(27– 41)	small to medium
		10	3	21	(19– 25)	few, small	—	38	(34– 43)	small to medium
30		—	3	68	(53– 93)	medium to large thecal hypertr.	+	109	(97–117)	estrous
		5	3	58	(43– 62)	medium to large	+	127	(118–144)	estrous
		10	3	40	(16– 80)	medium to large	+	121	(63–174)	estrous
		15	3	35	(26– 53)	medium to large	+	118	(86–145)	estrous
25	1.3	—	10	45	(36– 56)	medium to large	—	80	(56–110)	estrous
		5	5	37	(17– 64)	medium to large	—	74	(46–133)	estrous
		10	5	49	(57–100)	medium to large luteinized walls	1–5	79	(49–117)	estrous
		25	10	45	(21–150)	medium to large luteinized walls	—	85	(49–144)	estrous
		50	5	47	(25– 69)	medium to large luteinized walls	1–5	104	(64–150)	estrous
Hypophysectomized Controls			12	8	(6– 11)	small	—	23	(19– 32)	small

PMS.[5] The preparation of ICSH with MED 1.3 μg, when injected over the same range of dosage, was incapable of acting as an antagonist in the hypophysectomized rat. The reduction in ovarian weight observed after injection of the less purified preparations was due to depressed follicular development and increased atresia. The excessive luteinization which was a characteristic feature after injection of the high dosages persisted on administration of the purer ICSH preparations.

DISCUSSION

It appears that the antagonism shown by certain FSH-containing pituitary preparations is not an intrinsic property of FSH inasmuch as the phenomenon disappears during continued purification. It is therefore probable that the antagonism previously reported (11) (14) to be displayed by partially purified FSH preparations cannot be attributed to FSH itself.

The evidence given here also suggests that ultimately purified ICSH (LH) will not possess the intrinsic ability to antagonize endogenous or exogenous follicular stimulants. The burden of proof for a separate factor lies in the final purification of ICSH and/or recovery of a pituitary fraction

[5] Assayed against 20 I.U. PMS antagonism was exhibited by reduction of uterine as well as ovarian weight. After a primary gonadotrophic stimulus of 30 I.U. PMS ovarian weights were reduced progressively with increase in ICSH dosage; uterine weights were not reduced, in keeping with the common observation that uterine weights offer a less reliable index of antagonism in the presence of excess stimulation.

with antagonistic and no other biological property. It still remains a possibility that antagonism is due to some combination of known pituitary hormones, none of which is effective when administered alone.

The mechanism of action of antagonism remains to be elucidated. It is true that the end result of antagonism is limitation of follicular growth. The demonstration of the phenomenon in hypophysectomized animals has shown that pituitary mediation is not obligatory, although pituitary secretion of antagonist may be a contributory factor in intact animals. At least part of the reaction observed in normal rats may also be due to inhibition of secretion of endogenous FSH (13). Attention has been called to the similarity of the end results after injection of antagonist and after administration of estrogen.

The significance of the route of administration in demonstration of the phenomenon, i.e., the superiority of intraperitoneal injection over subcutaneous injection, remains to be explained. Differences in the rate of absorption and a possible role of the liver immediately come to mind.

ADDENDUM. PURIFICATION OF FSH AND ICSH

In order to evaluate the experiments presented here, it is essential to have a clear understanding of the degree of purity and the biological potencies of the preparations injected during these studies and to be able to compare them with others previously used in efforts to understand the phenomenon of antagonism. Stated briefly, the plan for the purification of anterior hypophyseal gonadotrophins of sheep has been as follows. Whole sheep pituitary glands have been extracted for FSH and ICSH with 40% ethanol, and for ICSH with aqueous solution, pH 9. Using partially purified hormones obtained by ammonium sulfate fractionation of these extracts, additional purification has been achieved by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose. The conditions have been carefully standardized for the best yield and potency of the gonadotrophins.

FSH

As shown in Table 5, preparations of FSH with MED 2.9 μg were obtained from ammonium sulfate fractionation followed by anion-exchange

TABLE 5. PURIFICATION OF SHEEP FOLLICLE STIMULATING HORMONE

Procedure	Yield	MED FSH, μg, SC	Multiple IT repair, IP
	mg./kg. wet. gl.		
Frozen pituitary	250×10^3	2500	0.4
Acetone-dried			
40% EtOH, pH7, 25° C	8000	250	1
$(NH_4)_2SO_4$ Fractionation	300–400	13–15	5
DEAE-Cellulose	30–40	2.9	35
$(NH_4)_2SO_4$, 2.0–2.4M	12–15	1.7	70

chromatography on DEAE-cellulose (0.5 milliequivalent ionizing groups per gram, equilibrated with 0.005M PO_4, pH 7.0) and displacement between 0.030M and 0.060M PO_4. When injected intraperitoneally, this FSH preparation had an MED for repair of interstitial tissue in hypophysectomized female rats at 35 times the MED for FSH. The reduced concentration of ICSH in the FSH-containing zone indicated partial separation of ICSH from FSH. Further fractionation of this FSH from DEAE-cellulose chromatography, by precipitation from 0.025% protein solution between 2.4 and 2.9M $(NH_4)_2SO_4$, yielded FSH with 1.7 μg MED. The presence of ICSH was not detected until this product was injected intraperitoneally at 70 times the MED for FSH. Table 6 shows the comparative

TABLE 6. FOLLICLE STIMULATING HORMONE FROM SHEEP PITUITARIES (FSH)
Comparison of products from different laboratories

Preparation		Unitage			Multiple of MED giving	
		MED μg F in H ♀ SQ	Multiple* of		Repair IT in H ♀ IP	VP 100% increase in H ♂ SC
Author and year	Method		Armour Std.	NIH-FSH-S1		
Jensen, *et al.* (17) 1939	40% EtOH; $(NH_4)_2SO_4$	25	6		10	
Fraenkel-Conrat, *et al.* (31, 34) 1940	40% EtOH; $(NH_4)_2SO_4$	3	55		40	
Li, *et al.* (35, 36) 1949	Aq. extr.; $(NH_4)_2SO_4$	≤100	ca 1.4		≥20	
Raacke, *et al.* (37) 1958	Electrophoresis	25	6			50
Ellis (23) 1958	NIH-FSH-S1; aq. extr.; $(NH_4)_2SO_4$	≤50	2.7		5	
	DEAE-C; Electrophoresis	4 (8)	20 (40)			35
Steelman and Segaloff (38, 39) 1957	EtOH; DEAE-C	ca 4	35			
Woods and Simpson (22, 40) 1959	40% EtOH; $(NH_4)_2SO_4$	14	10		5	20
	DEAE-C; $(NH_4)_2SO_4$	1.7	65	32	>70	85

* By augmentation bioassay in normal immature Long-Evans female rats using the conditions of Steelman and Pohley (41).

potency of this final preparation with others derived from sheep pituitaries, which have been described previously.

The methods used for purification of the sheep FSH used in these studies have been reported more fully in a recent publication by Woods and Simpson (22). Some more recent improvements have been made in the conditions of DEAE-cellulose chromatography. Using as starting material partially purified FSH from ammonium sulfate fractionation of 50% ethanol extract of sheep pituitaries, inert protein and most of the contaminating ICSH were removed by gradient displacement to 0.020M PO_4, pH 7.0, followed by gradient displacement to 0.040M PO_4 of the main FSH fraction. Such preparations are more potent, though at the sacrifice of yield. (For example, one fraction, with MED for FSH 2.3 μg and MED for ICSH 50 μg, had a yield of 15 mg./kg. wet weight. Judged

by augmentation bioassay (41) in normal immature female rats, this preparation had 45 times the FSH potency of NIH-FSH-S1. Another fraction, with MED for FSH 2.0 μg, and MED for ICSH 35 μg, had a yield of 14 mg./kg. wet weight.)

ICSH

Anion-exchange chromatography (38) on DEAE-cellulose of partially purified ICSH (MED 15 μg) from $(NH_4)_2SO_4$ fractionation of aqueous extracts of pituitary yielded ICSH with MED 3 μg. This preparation did not produce follicular or uterine stimulation at 1200 times the MED for ICSH when tested by subcutaneous injection in hypophysectomized rats. Subsequent cation-exchange chromatography on IRC-50, or fractionation with $(NH_4)_2SO_4$ between the limits of 1.4 and 1.8M, yielded ICSH with MED 2 μg. The yield was 45–50 mg./kg. wet weight of tissue, representing 35–40% recovery of activity in the original tissue. DEAE-cellulose chromatography of ICSH with MED 5 to 10 μg. derived by ammonium sulfate fractionation of 40% ethanol extracts, or a preparation with MED 2 μg from 50% ethanol extracts, resulted in ICSH with MED 0.75 to 1.0 μg. The yield of ICSH starting from 40 and 50% EtOH extracts was approximately 5 to 6 mg./kg. wet weight, or 1–2% recovery of activity in the original tissue. These preparations given subcutaneously did not cause follicular or uterine stimulation at 6000 times the MED for ICSH. The progressive increase in potencies and the yields from these fractionation procedures, applied to the aqueous and alcoholic starting material, are summarized in Table 7.

Table 8 shows the comparison with the results of other laboratories; only preparations from sheep pituitaries have been included since the products used in this study were only those derived from sheep pituitary.

ICSH preparations with potency of about 1 μg were assayed for biologically active contaminants at high doses in hypophysectomized rats and were found to contain less than 0.1% growth, lactogenic, adrenocortico-

TABLE 7. PURIFICATION OF SHEEP INTERSTITIAL CELL STIMULATING HORMONE

Extraction of glands	Progressive procedures	Yield, mg./kg. wet gl.	MED ICSH, μg IP	Multiple ♂ ♀ MED for F dev. SC
Aqueous, pH 9, 3° C	Frozen pituitaries	—	ca 5000	ca 2
	$(NH_4)_2SO_4$ 1.2–1.8M	1200–1500	15	125
	DEAE-Cellulose	200–400	3	>1200
	IRC-50 or $(NH_4)_2SO_4$ 1.4–1.8M	30–35	1–2	—
40% EtOH 25° C	Frozen pituitary	250×10^3	ca 1000	2.5
	Acetone-dried			
	40% EtOH, pH 7	8000	100	2.5
	$(NH_4)_2SO_4$ 1.4–1.8M	50–75	5–7.5	75
	DEAE-Cellulose	3–5	0.75–1.0	>6000

trophic or follicle-stimulating hormones. The MED for thyrotrophic hormone was 500 to 1000 μg. The presence of Proteinase I was demonstrable only on prolonged incubation for 6 to 24 hours. Contamination with vasopressin was less than 0.4 pressor units per mg.

TABLE 8. SHEEP INTERSTITIAL CELL STIMULATING HORMONE (ICSH OR LH)
Comparison of products from different laboratories

Preparation		Unitage (μg)		Multiple of Armour Std. 227–80		Multiple ♂ ♀ MED for F dev. SC
Author and year	Method	IT repair ♂ ♀ IP	VP* 100% SC	IT rep	VP	
Li, Simpson, Evans (42, 43) 1940	40% EtOH; $(NH_4)_2SO_4$	7	7			500
Squire, Li (44) 1958	H_2O extr.; IRC-50	2	≤ 2	2.5	≥7	>1000
Ellis (23) 1958	H_2O extr.; IRC-50	ca 5	5	1	3	150
Leonora, McShan, Meyer (45) 1958	IR-4B; IRC-50		<60			> 20
Woods, Simpson (40) 1959	H_2O extr.; $(NH_4)_2SO_4$; DEAE-C	3	5	1.7	5	>1200
	IRC-50; $(NH_4)_2SO_4$ (from above)	1–2		2.5	7	
	40% EtOH; $(NH_4)_2SO_4$; DEAE-C	0.75–1	3	4	5	≥6000

* Conditions of Greep *et al.* (46–47).

REFERENCES

1. EVANS, H. M. AND J. A. LONG: *Anat. Rec.* **21:** 62. 1921.
2. SMITH, P. E.: *J. Am. Med. Assoc.* **88:** 158. 1927.
3. EVANS, H. M. AND M. E. SIMPSON: *J. Am. Med. Assoc.* **91:** 1337. 1928.
4. LEONARD, S. L.: *Proc. Soc. Exper. Biol. and Med.* **31:** 1157. 1934.
5. DINGEMANSE, E. AND J. FREUD: *Acta brevia Neerl.* **9:** 157. 1939.
6. REISS, M., H. SELYE AND J. BALINT: *Endokrinologie* **8:** 15. 1931.
7. EVANS, H. M., M. E. SIMPSON AND P. K. AUSTIN: *J. Exper. Med.* **58:** 545. 1933.
8. LEONARD, S. L., F. L. HISAW AND H. L. FEVOLD: *Proc. Soc. Exper. Biol. and Med.* **33:** 319. 1935.
9. EVANS, H. M., K. KORPI, R. I. PENCHARZ AND M. E. SIMPSON: *Univ. of Calif. Publ. in Anat.* **1:** 237. 1936.
10. EVANS, H. M., M. E. SIMPSON, S. TOLKSDORF AND H. JENSEN: *Endocrinology* **25:** 529. 1939.
11. FRAENKEL-CONRAT, H., M. E. SIMPSON, C. H. LI AND H. M. EVANS: *Anales Fac. de Med. de Montevideo* **25:** 169. 1940.
12. FRAENKEL-CONRAT, H., C. H. LI, M. E. SIMPSON AND H. M. EVANS: *Endocrinology* **27:** 793. 1940.
13. GREEP, R. O., H. B. VAN DYKE AND B. F. CHOW: *Endocrinology* **30:** 635. 1942.
14. TALBERT, G. B., F. DIPILLO AND L. GORDIS: *Endocrinology* **61:** 611. 1957.
15. LEONARD, S. L.: *Am. J. Physiol.* **98:** 406. 1931.

16. Bischoff, F.: *J. Biol. Chem.* **133:** 621. 1940.
17. Jensen, H., M. E. Simpson, S. Tolksdorf and H. M. Evans: *Endocrinology* **25:** 57. 1939.
18. Fevold, H. L. and V. M. Fiske: *Endocrinology* **24:** 823. 1939.
19. Reiss, M., H. Selye and J. Balint: *Endokrinologie* **9:** 81. 1931.
20. Evans, H. M. and M. E. Simpson: *Anat. Rec.* **32:** 206. 1926.
21. Simpson, M. E., H. M. Evans and C. H. Li: *Proc. Soc. Exper. Biol. and Med.* **51:** 318. 1942.
22. Woods, M. C. and M. E. Simpson: *Endocrinology* **66:** 575. 1960.
23. Ellis, S.: *J. Biol. Chem.* **233:** 63. 1958.
24. Ellis, S., G. Noda, M. E. Simpson and H. M. Evans: *J. Biol. Chem.* **209:** 779. 1954.
25. Evans, H. M., M. E. Simpson, W. Marx and E. Kibrick: *Endocrinology* **32:** 13. 1943.
26. Greenspan, F. S., C. H. Li, M. E. Simpson and H. M. Evans: *Endocrinology* **45:** 455. 1949.
27. Fels, I. G., M. E. Simpson and H. M. Evans: *J. Biol. Chem.* **213:** 316. 1955.
28. Fraenkel-Conrat, J., H. Fraenkel-Conrat, M. E. Simpson and H. M. Evans: *J. Biol. Chem.* **135:** 199. 1940.
29. Rosenberg, L. L., E. S. Evans and M. E. Simpson: *Endocrinology* **68:** 1. 1961.
30. Adams, E. and E. L. Smith: *J. Biol. Chem.* **191:** 651. 1951.
31. Fraenkel-Conrat, H. L., M. E. Simpson and H. M. Evans: *Proc. Soc. Exper. Biol. and Med.* **45:** 627. 1940.
32. Walker, H. M. and J. Lev: *Elementary statistical methods.* Henry Holt and Co., N.Y., 1958. p. 143.
33. Fevold, H. L., F. L. Hisaw and R. Greep: *Am. J. Physiol.* **114:** 508. 1936.
34. Fraenkel-Conrat, H., M. E. Simpson and H. M. Evans: *Anales Fac. de Med. de Montevideo* **25:** 159. 1940.
35. Li, C. H., M. E. Simpson and H. M. Evans: *Science* **109:** 445. 1949.
36. Li, C. H. and K. O. Pedersen: *J. Gen. Physiol.* **35:** 629. 1952.
37. Raacke, I. D., A. J. Lostroh and C. H. Li: *Arch. Biochem. Biophys.* **77:** 138. 1958.
38. Steelman, S. L. and A. Segaloff. *Abstract, Endocrine Society 39th Meeting, N.Y.* No. **158:** 104. 1957.
39. Steelman, S. L. and A. Segaloff: *Rec. Prog. Hormone Res.* **15:** 115. 1959.
40. Woods, M. C. and M. E. Simpson: *Fed. Proc.* **18:** Pt. 1: 173. 1959.
41. Steelman, S. L. and F. M. Pohley: *Endocrinology* **53:** 604. 1953.
42. Li, C. H., M. E. Simpson and H. M. Evans: *Science* **92:** 355. 1940.
43. Li, C. H., M. E. Simpson and H. M. Evans: *Endocrinology* **27:** 803. 1940.
44. Squire, P. G. and C. H. Li: *J. Biol. Chem.* **234:** 520. 1959.
45. Leonora, J., W. H. McShan and R. K. Meyer: *Endocrinology* **63:** 867. 1958.
46. Greep, R. O., H. B. Van Dyke and B. F. Chow: *Proc. Soc. Exper. Biol. and Med.* **46:** 644. 1941.
47. Lostroh, A. J., P. G. Squire and C. H. Li: *Endocrinology* **62:** 833. 1958.

FURTHER STUDIES OF FACTORS AFFECTING THE PLASMA PROTEIN-THYROID HORMONE COMPLEX[1,2]

MILTON W. HAMOLSKY, MYRON STEIN, DIANA B. FISCHER AND A. STONE FREEDBERG

Department of Medicine, Harvard Medical School, and the Medical Research Department of the Yamins Research Laboratory and the Medical Service, Beth Israel Hospital, Boston, Massachusetts

ABSTRACT

The binding of T4 and T3 by plasma proteins and their resultant distribution between plasma and tissue binding sites were studied by (1) paper and starch gel electrophoresis, (2) *in vitro* tissue uptakes, and (3) dialysis. Results indicate that (1) the plasma inter-alpha globulin binding moiety of paper electrophoresis is heterogeneous on reelectrophoresis in starch gel, (2) the T4 binding component of serum albumin resides in a very small fraction thereof, (3) TRIS buffer plays an important role in plasma binding of T4 in these techniques, (4) pCO_2 plays an important role in T4 and T3 binding by plasma, and (5) pH affects the binding of thyroid hormones and this effect is different for T4 and T3.

RECENT reviews attest to the growing interest in, and increasing complexity of, the nature of the binding and transport of thyroid hormonal substances by various plasma protein components (1–4). In early studies, using chiefly paper electrophoresis in Veronal buffer, pH 8.6, it was found that thyroxine (T4) migrated principally with a trace, inter-alpha globulin moiety (TBP or TBG), with albumin serving as a secondary carrier of a small proportion of thyroxine. More recently, another important thyroxine-binding plasma component—a pre-albumin fraction (TBPA) has been found by several investigators using different buffers at pH 8.6 and varied electrophoretic techniques (5–11). Comparable data on the binding of triiodothyronine (T3) are meager, but it appears that (1) T3 is similarly bound, perhaps preferentially, to TBP, (2) T3 does associate with various other protein fractions on electrophoresis, (3) T3 binding by plasma protein is weaker than that of T4, and (4) T3 is not bound by pre-albumin (6, 10, 12, 13). This report deals with our

Received September 15, 1960.

[1] This investigation was supported by a research grant, A-140, from the National Institute of Arthritis and Metabolic Disease of the National Institutes of Health, Public Health Service.

[2] Based on material presented at the Fourth International Goiter Conference in London, England—July, 1960.

continuing study of factors affecting thyroid hormone binding by plasma proteins and the resultant distribution of hormone between plasma and tissue binding sites, using concomitantly methods of paper and starch gel electrophoresis, *in vitro* "uptakes," and dialysis. The results indicate further the importance, in this parameter of thyroid function, of such factors as the method of electrophoresis, the nature of the buffer, the specific thyroid hormone involved, the pH of the medium, and the pCO_2 of the system.

MATERIALS AND METHODS

Electrophoresis: Plasma or serum was labeled by the addition of varying amounts of I^{131} T4 or I^{131} T3 (resulting in added concentrations of 1–200 $\mu g\%$) and subjected, in different buffer systems, to (a) paper electrophoresis ("hanging" paper—Spinco model—Durrum type), (b) starch gel electrophoresis by the method of Smithies (14), and (c) varying combinations of paper and starch gel electrophoresis. In reference to (c) above, sequential 1 cm. segments of a paper strip after electrophoresis were inserted into a slit in starch gel and re-electrophoresed, using the same or a different buffer. Alternatively, the contents of various 1 cm. segments of the starch gel after electrophoresis were removed by freezing, thawing, and squeezing, and then reelectrophoresed on paper or on another starch gel using a different buffer. The distribution of radioactivity was determined by (a) direct strip or gel counting in an automatic, gas flow, end-window scanner, (b) by cutting the strip or gel into 1 cm. segments and counting the segments in a well-type scintillation counter, and (c) by radioautography (for paper strips only). For protein distribution, paper strips were stained by bromphenol blue, gels by the method of Smithies. The distribution of radioactivity was estimated by planimetric scanning of the area under the various radioactive peaks.

In Vitro Uptake Studies: Using our previously published method (15), tracer amounts of I^{131} T3 or I^{131} T4 were added to aliquots of whole blood, incubated with shaking at 37° C for varying time intervals, and the incorporation of radioactivity by the red blood cells then determined following five saline washes. R.b.c. uptake results are expressed as the % uptake, viz.:

$$\frac{\text{counts/minute in r.b.c.} \times 100}{\text{total counts in medium/minute}}$$

and the answer corrected to 100 hematocrit.

Dialysis Studies: The amount and rate of dialysis of I^{131} T4 and I^{131} T3 from *in vitro* labelled plasma or serum were determined according to our previously published method (10).

In the studies on the role of carbon dioxide tension, the pCO_2 of various solutions was determined both by the Van Slyke apparatus and by a highly sensitive CO_2 electrode (Severinghaus type).

RESULTS

Following paper electrophoresis of I^{131} T4 labeled serum in Veronal buffer (pH 8.6, 0.1M), the radioactivity was found, as reported by others, principally in the inter-alpha globulin area, with a small amount in the proximal region of the broad albumin band (Fig. 1, upper portion). However, when the paper segment containing the albumin-localized T4 was re-electrophoresed on starch gel (borate buffer, pH 8.6, 0.3M) the albumin

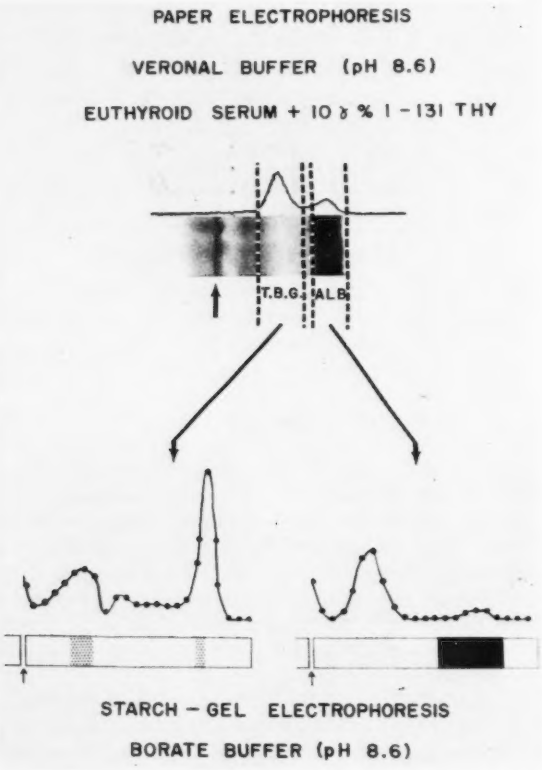

FIG. 1. Combination of paper (upper section) and starch gel (lower two sections) electrophoresis. Upper section—whole euthyroid serum; lower sections—reelectrophoresis of albumin segment from paper (right), of TBG segment (left). In each section, upper portion represents scan or plot of radioactivity, lower portion depicts protein distribution. Arrows indicate point of application of serum or insertion of paper segment into starch gel.

segment was resolved into a major, broad, protein staining peak with little radioactivity and a narrow band closer to the origin containing most of the radioactivity but not stainable protein (Fig. 1, bottom right). Similarly, as seen in the lower left section of Figure 1, the TBG segment from paper, when re-electrophoresed on starch gel, revealed two and occasionally three distinct bands of radioactivity with faint protein stains.

The pattern of distribution of radioactivity obtained on starch gel electrophoresis of T4-labeled serum in Veronal buffer was similar to that on paper—namely, a major inter-alpha globulin peak and a smaller albumin peak. However, following starch gel electrophoresis of I^{131} T4 labelled serum in TRIS (hydroxymethylaminomethane) buffer (pH 8.6,

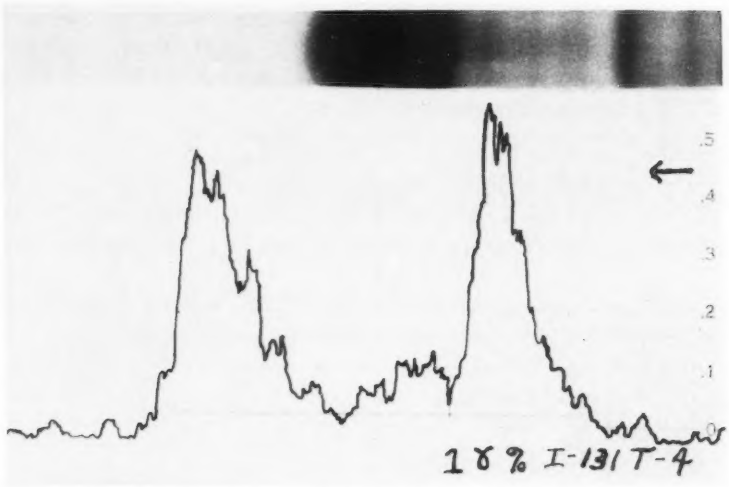

FIG. 2. Starch gel electrophoresis of euthyroid serum, labelled by addition of 1γ% I^{131} T4. TRIS buffer, pH 8.6, 0.5M. Migration from right border to left. Upper strip—protein stain; lower section—radioactivity scan.

0.5M), as seen in Figure 2, radioactivity was found in three peaks: 1) a leading pre-albumin band, 2) a small amount in the albumin band, and 3) a peak interpreted as representing the inter-alpha globulin area. No pre-albumin localization of I^{131} T3 could be demonstrated. The ratio of the amount of T4 (4 μg% added) in the TBP peak as compared to the TBPA peak was then compared in repeated determinations in nine euthyroid and ten untreated hyperthyroid patients (Table 1). No consistent pattern

TABLE 1. PERCENT DISTRIBUTION OF I^{131}-I-THY BETWEEN PRE-ALBUMIN AND INTER-ALPHA GLOBULIN ON STARCH GEL ELECTROPHORESIS—(TRIS BUFFER, PH 8.6, 0.5M)

Euthyroid				Hyperthyroid			
Patient (No. of runs)	TBP*	TBPA†	Ratio TBP / TBPA	Patient (No. of runs)	TBP*	TBPA†	Ratio TBP / TBPA
1 (2)	30.4	55.2	0.55	1 (2)	14.0	67.5	0.21
2 (2)	31.9	52.8	0.60	2 (2)	29.3	51.3	0.57
3 (3)	32.9	53.8	0.61	3 (3)	37.6	43.3	0.87
4 (3)	35.3	48.7	0.72	4 (2)	38.3	43.8	0.87
5 (2)	41.1	47.8	0.86	5 (2)	39.7	49.8	0.80
6 (2)	44.0	40.2	1.09	6 (2)	39.8	49.0	0.81
7 (5)	45.2	37.2	1.22	7 (2)	41.6	39.9	1.04
8 (4)	53.3	29.6	1.80	8 (2)	48.2	33.8	1.43
9 (5)	54.4	32.2	1.69	9 (2)	56.2	23.2	2.42
				10 (3)	58.5	29.9	1.95

* TBP = Inter-Alpha Globulin.
† TBPA = Prealbumin.

of distribution could be demonstrated as there was complete overlapping of the values for the ratio of distribution

$$\frac{(\%\ \text{T4 in TBP})}{\%\ \text{T4 in TBPA}}$$

between the euthyroid and hyperthyroid series. In contrast to the results reported using TRIS buffer on paper electrophoresis, the ratio of distribution of T4 between the TBPA and TBP in a given serum could be varied, even reversed, by altering the molarity of the TRIS employed in the system.

These and previously observed effects of TRIS (10) and its known capacity as a CO_2 buffer refocused our attention on anomalous results we had

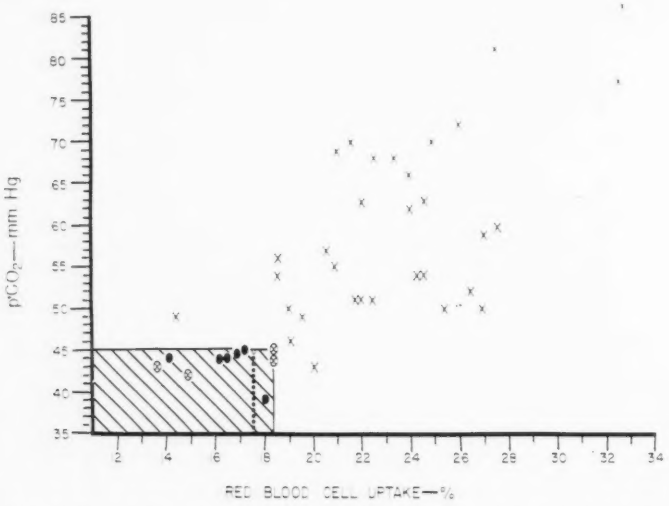

FIG. 3. Correlation of 2-hour *in vitro* r.b.c. uptake of I^{131} T3 in whole blood vs. pCO_2. (See text for details.)

been observing in our previous studies on the *in vitro* distribution of T3 between plasma and red blood cells in patients with CO_2 retention (17). In Figure 3 are plotted 32 r.b.c. uptakes (abscissa) in 22 consecutive patients with CO_2 retention (ordinate). Nineteen had chronic pulmonary emphysema, two kyphoscoliosis, one marked hypoventilation following cerebrovascular accident. The hatched areas indicate normal values in our laboratories (15, 17). The dotted vertical line represents the upper limit of normal of the two hour *in vitro* r.b.c. uptake of I^{131} T3 for females (17.0%) and the solid line that for males (18.5%). A highly significant correlation between pCO_2 and r.b.c. uptake is observed, with an r value of 0.94. The solid circles represent normal uptakes in six patients with severe dyspnea

from obstructive pulmonary emphysema but with normal pCO_2 values. The circled crosses represent results in four patients with previously elevated values whose uptake fell to normal as pCO_2 fell in response to therapy. The elevated uptake depends upon a plasma abnormality as red blood cells from a normal subject had elevated uptakes in hypercapneic plasma, whereas cells from a hypercapneic patient had normal uptakes in normal plasma (Fig. 4). Blood pH was normal in most patients while slightly reduced (7.34 to 7.38) in the remaining few at the time of these determinations.

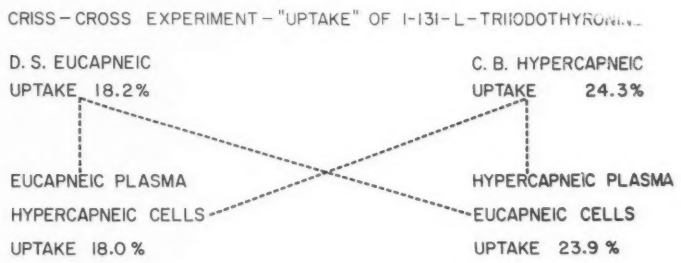

FIG. 4. Effect of recombination of eucapneic plasma with hypercapneic cells and hypercapneic plasma with eucapneic cells on 2-hour *in vitro* r.b.c. uptake of I^{131} T3.

I^{131} T4 turnover studies were carried out in seven of these hypercapneic patients following the infusion of 25 microcuries of I^{131} T4. Normal values in our laboratory range from 6.6 to 7.8 days with an average of 7.18 days (19). In contrast, the half-time of disappearance of the infused I^{131} T4 in the seven hypercapneic patients ranged from 4.2 to 5.8 days, averaging 5.1 days.

We have reproduced some of these phenomena experimentally. The red cell uptake of I^{131} T4 or I^{131} T3 is decreased up to 50% *in vitro* if pCO_2 is permitted to fall, as by incubating in an open flask. The uptake can be returned to normal simply by restoppering the flask and continuing the run or by maintaining normal pCO_2 during the run by passing CO_2 into the unstoppered flask. R.B.C. uptake can be increased above normal by bubbling in CO_2 under pressure. It is noteworthy, however, that to date we have not been able to obtain uptake values as high as those obtained in the hypercapneic patients. Normal uptakes, once attained, decrease if the flask is then unstoppered and the run continued. Similarly, the dialysis of T4 or T3 from plasma to a buffer medium can be decreased or increased, respectively, by decreasing or increasing the surrounding pCO_2. The increased dialysis was observed following the increased pCO_2 despite maintenance of pH within a narrow range by the concomitant addition of bicarbonate ion.

Other studies, however, revealed a separate role of pH in affecting hor-

mone binding. The dialysis of T4 and T3 from plasma to various buffers was determined over an extended pH range. As seen in Figure 5, the plasma binding of T3 progressively increased (dialysis decreased) at progressively higher pH values. In contrast, the binding of T4 was essentially unchanged between pH's of 7.3 and 8.3, but decreased at pH 8.6.

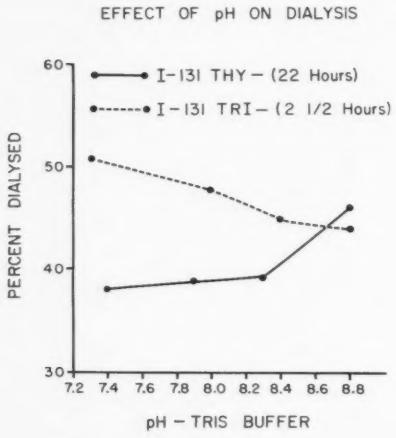

FIG. 5. Effect of pH on dialysis of I^{131} T4 and I^{131} T3 (1.0 μg %) from plasma to TRIS buffer.

DISCUSSION

Knowledge concerning the binding and transport of thyroid hormones by one or more plasma proteins has grown at a rapid pace. Important physiologic correlations have been made at varying stages of development of this knowledge (e.g. increased binding capacity has been shown in myxedema, pregnancy, and following estrogen administration, while a decreased binding capacity has been noted in hyperthyroidism, nephrosis, etc.). The demonstration of an important degree of T4 binding by a pre-albumin factor in different buffers at pH 8.6 by various electrophoretic techniques (5–11) has necessitated a reevaluation of earlier concepts based on the assumption of only one major (TBG) and one lesser (albumin) binding protein. The unsettled state of the problem is reflected by the different reports that 1) on starch gel electrophoresis in borate buffer the major binding of T4 occurs in a pre-albumin fraction (5). 2) on paper electrophoresis in TRIS buffer there is an approximately equal distribution of T4 between TBPA and TBG at physiological concentrations of T4 (6), 3) the T4 binding properties of TBG are due to TBPA present as a complex of unknown composition (7), 4) four binding sites of T4 have been identifield on starch gel electrophoresis and radioautography (19), 5) an iodine compound is found associated with albumin in the plasma of certain thyrotoxic but not euthyroid subjects (20) and 6) the distribution of T4 on paper electrophoresis in TRIS and borate buffer is strikingly pH dependent (11).

At pH 8.6, the distribution between TBPA, albumin, and TBG averaged 56, 8, and 34%, respectively; at the more physiological pH of 7.6, however, there was essentially no binding to pre-albumin, although a potential T4 binding component ahead of albumin could be demonstrated in two-dimensional electrophoresis. Such reports and the results of this study indicate the need for critical evaluation of the role of the various experimental techniques employed in attempting any physiologic assessment of this parameter of thyroid function.

The results of our study using various combinations of paper and starch gel electrophoresis indicate a heterogeneity of the inter-alpha globulin binding protein. The single TBG peak found on paper was consistently resolved into two and sometimes three distinct peaks on starch gel re-electrophoresis. Furthermore, the specific binding moiety of the albumin band was shown on re-electrophoresis to reside in but a very small portion thereof. Such data are pertinent to attempted mathematical computation of T4 binding, using e.g. chemical methods to quantitate albumin concentrations. They further underscore the fact that the binding components have not yet been isolated in pure form, cannot be quantitated directly in different sera, and, therefore, it is not possible at this date to determine whether an alteration in T4 distribution in various disease states represents a quantitative increase or decrease of a binding protein or some form of qualitative alteration thereof.

The importance of technical factors such as the buffer used is further indicated by our published observations (10) that (1) binding capacity of TBPA isolated in a starch gel, TRIS system is dependent upon the presence and molarity of the TRIS buffer, (2) TRIS buffer causes an increased plasma binding of T4 in the *in vitro* erythrocyte uptake and dialysis techniques and (3) Veronal buffer causes a striking decrease of the binding of T4 by albumin.

The possible role of carbon dioxide tension in affecting the distribution of thyroid hormones between plasma and tissue binding sites is suggested by (1) the striking correlation of pCO_2 levels and *in vitro* r.b.c. uptakes of I^{131} TRI, (2) the increased T4 turnover *in vivo* in hypercapneic subjects, (3) the correlation of *in vitro* tissue uptakes with experimentally altered pCO_2 levels, and (4) the correlation of the rates of dialysis of T4 and T3 from plasma with experimentally altered pCO_2 levels. It is not possible at this time to delineate a unique role for pCO_2 independent of the concomitant pH changes or to define the mechanism of the pCO_2 effect. However, a pCO_2 effect, separate from pH alteration, is suggested by the following: 1) the pH in the blood of the hypercapneic patients when the r.b.c. uptake was distinctly elevated was in a normal range or only slightly reduced, 2) a decrease in r.b.c. uptake correlated directly with therapeutically induced lowering of pCO_2, when attainable, without significant pH changes, 3) experimental prevention of alteration of the pH to any

significant degree by the concomitant addition of bicarbonate failed to prevent the increased rate of dialysis attendant upon increased pCO_2 tensions, 4) although the decreased red cell uptake of I^{131} TRI effected by running the experiment in an open flask (with the attendant increased pH) could be reversed to normal by simply restoppering the flask and continuing the run, the elevated pH did not return to the lower control values *pari passu*, and 5) although a decreased pCO_2 (with an increased pH) caused a decreased *in vitro* r.b.c. uptake of *both* T3 and T4, and an increased pH alone caused a decreased dialysis of T3 from plasma (consistent with an increased plasma binding, hence a decreased tissue uptake), an increased pH caused an *increase* in the dialysis of T4 which would suggest decreased plasma binding and an *increased* tissue uptake if the pH were the determinant factor.

Further studies are under way to investigate the possible physiologic role of pCO_2 and pH alterations in the distribution of thyroid hormones between plasma and tissue binding sites.

REFERENCES

1. Robbins, J. and J. E. Rall: *Recent Prog. Hormone Res.* **13:** 161. 1957.
2. Hamolsky, M. W. and A. S. Freedberg: *N.E.J.M.* **262:** 23. 70. 129. 1960.
3. Ingbar, S. H.: In *Clinical Endocrinology*, edited by E. B. Astwood. pp. 91–102. Grune and Stratton, New York and London, 1960.
4. Tata, J. R.: *Brit. med. Bull.* **16:** 142. 1960.
5. Rich, C. and A. G. Bearn: *Endocrinology* **62:** 687. 1958.
6. Ingbar, S. H.: *Endocrinology* **63:** 256. 1958.
7. Tata, J. R.: *Nature* **183:** 877. 1959.
8. Beierwaltes, W. H. and J. Robbins: *J. Clin. Investig.* **38:** 1683. 1959.
9. Andreoli, M. and D. Andreani: L'elettroforesi continua applicata allo studio degli ormoni radioattivi. Commun. III Congr. Soc. It. Biol. Med. Nucleare, Firenze, Nov. 1958.
10. Hamolsky, M. W., H. A. Ravin and A. S. Freedberg: *Endocrinology* **66:** 780. 1960.
11. Myant, N. B. and C. Osorio: *J. Physiol.* **152:** 601. 1960.
12. Deiss, W. P., E. C. Albright and F. C. Larson: *Proc. Soc. Exper. Biol. and Med.* **84:** 513. 1953.
13. Dingledine, W. S., R. Pitt-Rivers, and J. B. Stanbury: *J. Clin. Endocrinol. and Metab.* **15:** 724. 1955.
14. Smithies, O.: *Biochem. J.* **61:** 629. 1955.
15. Hamolsky, M. W., M. Stein and A. S. Freedberg: *J. Clin. Endocrinol. and Metab.* **17:** 33. 1957.
16. Hamolsky, M. W., H. A. Ravin and A. S. Freedberg: Unpublished observations.
17. Hamolsky, M. W., A. Golodetz and A. S. Freedberg: *J. Clin. Endocrinol. and Metab.* **19:** 103. 1959.
18. Kurland, G. S., J. G. Bustos, M. W. Hamolsky, and A. S. Freedberg: *J. Clin. Endocrinol. and Metab.* **17:** 1365. 1957.
19. Robbins, J. and J. E. Rall: Personal communication. Data presented at Fourth International Goiter Conference, London, England, July, 1960.
20. Cameron, C. and K. Fletcher: *Nature* **183:** 116. 1959.

THE MECHANISM OF IODINATION REACTIONS IN THYROID GLANDS[1]

NICHOLAS M. ALEXANDER

Radioisotope Service, Veterans Administration Hospital, West Haven, Connecticut and the Department of Biochemistry, Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

Previous studies from this laboratory showed that inorganic iodide is oxidized by H_2O_2 and an enzyme, iodide peroxidase, before it is incorporated into tyrosine and protein by thyroid and salivary homogenates. It is reported here that several other naturally occurring biological oxidants or electron carriers cannot replace H_2O_2, although it had been shown elsewhere that flavin cofactors stimulate iodide utilization by thyroid preparations. The results of this study demonstrate that catalase or anaerobiosis prevent flavin activation, and thus support the conclusion that the activation is due to the production of H_2O_2 during autoxidation of the flavin. The peroxidases of salivary and thyroid glands are not identical because a homogenate of salivary gland will oxidize bromide and slightly incorporate it into tyrosine, but thyroid homogenates will not. Neither tissue oxidizes chloride or fluoride. Histidine is iodinated by thyroid and salivary extracts but tyrosine is overwhelmingly iodinated when both amino acids are present. Although iodohistidine has been isolated from thyroid glands, it has not previously been biosynthesized *in vitro*. Under conditions where tyrosine is readily iodinated, possible thyroxine precursors, thyronine, di- and tri-iodothyronine, are not iodinated by rat or human thyroid extracts. A suggestion for the *in vitro* role of Cu^{++} in iodine metabolism is presented.

RECENT work by the author (1) demonstrated that the enzyme catalyzing iodide oxidation in the thyroid is a peroxidase, hydrogen peroxide being the oxidizing agent (2). Reports (3, 4) that riboflavin or riboflavin phosphate stimulate radioiodide incorporation into the protein of a rat thyroid subcellular particulate preparation indicated that iodide may have been oxidized by something other than iodide peroxidase. This investigation shows that stimulation is due to the generation of hydrogen peroxide by autoxidation of the flavin. Consistent with the fact that iodide peroxidase is the oxidative catalyst in thyroid tissue, several other electron carriers cannot replace hydrogen peroxide. Differences in the halide

Received September 15, 1960.

[1] This paper was awarded the 1960 Van Meter Prize by The American Goiter Association.

specificities of the iodide peroxidases of thyroid and salivary tissue and studies on the iodination of histidine and iodothyronine derivatives are also presented.

METHODS

Tissue Homogenates, Materials, Incubation Procedures, and Manometry. Homogenates of rat thyroid and submaxillary salivary glands were incubated aerobically (1) with 0.1 to 0.3 μc of radioiodide in sodium phosphate buffer (pH 7.4, 0.1 M) in a Dubnoff shaker or in a Warburg apparatus. Whole thyroid glands could be stored frozen for 3 months without losses in enzymatic activity. Anaerobic incubations were shaken at 37° in Thunberg tubes that were evacuated with a water aspirator for ten minutes or were filled with nitrogen. Incubations containing riboflavin were exposed to the prevailing light in the laboratory.

Monoiodohistidine and 3-monobromotyrosine were synthesized (5, 6) for use as markers on paper chromatograms and all other materials were obtained from commercial sources.

Separation of Iodoamino Acids and Radioiodine Assays. Incubations were terminated with trichloroacetic acid and iodoamino acids in the supernatant solution were isolated by chromatography on Dowex-50 and paper and counted as described (1). The thyroid protein precipitates were counted after being washed three times with 0.1 mg. of carrier potassium iodide in 2 ml. of 5% trichloroacetic acid to remove non-protein bound radioiodine. When less than 10^{-4}M I^{131}-iodide was the substrate, carrier iodoamino acids (20 to 50 μg) were added to the paper chromatograms to reveal their location with the Pauly reagent.

RESULTS

Inhibition of Riboflavin Stimulation by Catalase or Anaerobiosis. Table 1 contains typical data demonstrating that riboflavin stimulates the incorporation of radioiodide into L-tyrosine and protein by thyroid homogenates. Only MIT[2] was synthesized and most of the radioactivity in the pancreatin hydrolystes (7) of the proteins was also present as MIT. In the absence of added tyrosine, somewhat more iodide is fixed into protein. Riboflavin stimulation is profoundly inhibited by one mg. of catalase, but boiled catalase has no effect.

TABLE 1. INHIBITION OF RIBOFLAVIN STIMULATION BY CATALASE

Conditions: Each reaction flask was incubated for one hour and contained the following in a total volume of 1.5 ml.: 0.5 ml. rat thyroid homogenate, 3.3×10^{-5} M KI^{131}, 6.7×10^{-5} M riboflavin, and 3.3×10^{-3} M L-tyrosine. Substances were added or omitted as indicated.

Reaction flask	Constituent added or omitted	% of added iodide incorporated into:	
		Thyroid protein	MIT
1	No riboflavin	0.4	0.3
2	0	4.9	26.3
3	No tyrosine	9.0	1.0
4	One mg. catalase added	0.5	0.7
5	One mg. boiled catalase added	8.8	29.8

[2] The abbreviations employed are: MIT = 3-monoiodotyrosine; DIT = 3,5-diiodotyrosine; DPN = Diphosphopyridine nucleotide; and TPN = Triphosphopyridine nucleotide.

TABLE 2. INHIBITION OF RIBOFLAVIN STIMULATION BY ANAEROBIOSIS

Conditions: Same as reaction 2 in Table 1 except that the main compartment of the Thunberg tubes contained everything but the homogenate which was tipped in from the side arm after anaerobiosis had been achieved. Tube 1 was also evacuated, then reaerated, before tipping in the homogenate and Tube 3 was filled with nitrogen after it had been evacuated.

Thunberg tube No.	Gas phase	% of added iodide incorporated into:	
		Thyroid protein	MIT
1	Air	5.3	27.8
2	Anaerobic	0.2	0.4
3	Nitrogen	0.5	0.8

From the data in Table 2 it is obvious that anaerobiosis completely prevents riboflavin stimulation of iodide utilization by thyroid homogenates. The control incubation, tube 1, was evacuated in the same manner and reaerated to establish that manipulations for achieving anaerobiosis were not responsible for reversal of the stimulatory effect. Identical results to those in Table 1 and 2 were obtained when riboflavin phosphate was substituted for riboflavin. The stimulation by flavins of iodide utilization by rat salivary and human thyroid homogenates was also inhibited by catalase or anaerobiosis.

Inability of Other Oxidants and Electron Carriers to Replace Hydrogen Peroxide. The specific requirement for hydrogen peroxide in thyroid homogenates is illustrated in Table 3. Here it is seen that the hydrogen peroxide generating system, glucose and glucose oxidase, markedly enhances utilization of radioiodide by thyroid homogenates. Only MIT is formed with this concentration of iodide whereas previously it had been found that DIT was also formed with 50 times as much iodide (1). Some of the naturally abundant biological electron carriers and other widely used oxidants cannot replace hydrogen peroxide. The electron carriers

TABLE 3. INABILITY OF VARIOUS ELECTRON CARRIERS TO REPLACE H_2O_2 IN THE THYROID IODINATING SYSTEM

Conditions: All flasks were incubated for one hour and contained 0.3 ml. rat thyroid homogenate, 6.7×10^{-5} M KI^{131}, 3.3×10^{-3} M L-tyrosine, and the substances indicated below in a total volume of 1.5 ml. H_2O_2 was produced from 6.7×10^{-3} M glucose and 125 micrograms of glucose oxidase. Concentration of the other oxidants was 10^{-3} M except for cytochrome c and $K_3Fe(CN)_6$ whose concentrations were 3.7×10^{-4} M and 6.7×10^{-3} M respectively.

Reaction flask	Electron carrier or oxidant added	% of added iodide incorporated into:	
		Thyroid protein	MIT
1*	H_2O_2	1.0	0.8
2	H_2O_2	3.9	31.4
3	Methylene Blue	1.2	0.2
4	DPN	0.2	0.2
5	TPN	0.2	0.2
6	Cytochrome c	0.4	0.2
7	Potassium Ferricyanide	0.2	0.4
8	Phenazine Methosulfate	1.2	1.9

* Boiled thyroid homogenate employed.

which were ineffective are cytochrome c, DPN, TPN, ferricyanide, methylene blue, and phenazine methosulfate. Similar results were obtained with salivary, rat extraorbital lacrimal, and human thyroid homogenates. Thyroid, salivary and lacrimal homogenates also very actively incorporate iodide into tyrosine at pH 5.4 (0.1 M acetate buffer) when H_2O_2 is supplied.

Differences in the Halide Specificities of Iodide Peroxidases in Thyroid and Salivary Glands. It was previously established (1) that glucose oxidase consumes 1 mole of oxygen per mole of glucose in generating hydrogen peroxide and gluconic acid, but traces of metals in water and buffer salts decompose hydrogen peroxide to 1/2 O_2 and H_2O so that the net oxygen uptake is halved:

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{Oxidase}]{\text{Glucose}} H_2O_2 + \text{Gluconic Acid}$$

$$H_2O_2 \xrightarrow{\text{Metals}} H_2O + 1/2\ O_2$$

$$\text{Net: (1) Glucose} + 1/2O_2 \longrightarrow \text{Gluconic Acid}$$

When iodide and salivary or thyroid homogenate are added to this H_2O_2 generating system, the net oxygen uptake increases because H_2O_2 is not decomposed but is preferentially utilized by iodide peroxidase to oxidize iodide ion in the following manner:

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{Oxidase}]{\text{Glucose}} H_2O_2 + \text{Gluconic Acid}$$

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow[\text{Peroxidase}]{\text{Iodide}} 2\text{"Active I"} + 2H_2O$$

$$\text{Net (2) Glucose} + O_2 + 2I^- + 2H^+ \longrightarrow 2\text{"Active I"} + \text{Gluconic Acid} + H_2O$$

"Active I", perhaps iodinium ion I^+ (8), then iodinates tyrosine.

The activity of thyroid and salivary peroxidases towards various halogens was assessed in this manner (cf. ref. 1), and the results are expressed in Table 4. In accordance with equation (1), 5 μmoles of oxygen are consumed when 10 μmoles of glucose are oxidized by glucose oxidase (incubation 1) and, thyroid and salivary homogenates readily peroxidize iodide as seen by the increase in oxygen uptake (incubations 7 and 13). Positive tests for iodine with starch are observed in these iodide incubations. In the absence of thyroid or salivary homogenates, iodide and bromide are not significantly oxidized, incubations 2 and 3. Salivary (incubation 8), but not thyroid (incubation 12), homogenates also readily peroxidize bromide, while neither tissue peroxidizes chloride or fluoride, incubations 5, 6, 10 and 11. Preliminary results indicate that dialyzed lacrimal gland homogenates peroxidize bromide as well as iodide. (Lacrimal homogenates were dialyzed to reduce endogenous oxygen consumption to a minimum while dialysis of salivary and thyroid homogenates is not neces-

TABLE 4. DIFFERENCES IN HALIDE PEROXIDATIONS BY THYROID AND SALIVARY HOMOGENATES

Conditions: All incubation mixtures contained 10 μmoles of glucose, 125 μg glucose oxidase, and 10 μmoles L-tyrosine. 50 μmoles of halide and 1.5 ml. of salivary homogenate or 1.3 ml. of thyroid homogenate were added as indicated. Final volume was 3.0 ml. and oxygen uptake was at the end of 2 hours.

Incubation number	Homogenate added	Halide added	O_2 uptake* in μmoles
1	0	0	5.0
2	0	Iodide	5.2
3	0	Bromide	5.1
4	Salivary	0	5.3
5	Salivary	Fluoride	5.6
6	Salivary	Chloride	5.1
7	Salivary	Iodide	9.8†
8	Salivary	Bromide	9.6†
9	Thyroid	0	5.3
10	Thyroid	Fluoride	5.4
11	Thyroid	Chloride	5.7
12	Thyroid	Bromide	5.5
13	Thyroid	Iodide	8.8†

* Values were the averages of 3 determinations.
† Boiled homogenates were inactive.

sary.) Paper chromatograms of Dowex-50 effluents from incubation mixtures containing 0.0017 M KBr and salivary homogenates displayed a faint Pauly positive spot with the same Rf (0.55) as monobromotyrosine but no dibromotyrosine was apparent, whereas MIT and DIT are readily synthesized from 0.0017 M KI (1).

The effect of bromide on the incorporation of radioiodide into L-tyrosine and protein by salivary and thyroid homogenates is presented in Table 5. A fifty fold excess of bromide does not alter the incorporation of iodide into L-tyrosine by salivary tissue, and, as expected, bromide has no effect on the utilization of iodide by thyroid homogenates. A negligible percentage of iodide was incorporated into salivary protein because the iodide concentration was high (10^{-3} M) and tyrosine was added. Salivary homogenates effectively incorporate iodide into tyrosine when 1.67×10^{-3} M

TABLE 5. FAILURE OF BROMIDE TO INHIBIT IODIDE UTILIZATION BY SALIVARY AND THYROID HOMOGENATES

Conditions: Reaction flasks 1 to 4 contained 1.0 ml. salivary homogenate, 1.67×10^{-3} M KI^{131}, and H_2O_2 generated by 3.3×10^{-3} M glucose and 125 μg glucose oxidase in a total volume of 3.0 ml. Flasks 5 to 7 contained the same constituents except that salivary tissue was replaced by 0.5 ml. thyroid homogenate and KI concentration was 6.7×10^{-5} in a total volume of 1.5 ml. Bromide was added as indicated and the incubations lasted for 1 hour. The tyrosine concentration was 3.3×10^{-3} M in all flasks.

Reaction flask	Concentration of bromide added	% of added iodide incorporated into:		
		Protein	MIT	DIT
1—Salivary*	0	0.1	0.9	0
2—Salivary	0	0.2	44.6	25.4
3—Salivary	Equivalent to iodide	0.2	43.2	24.5
4—Salivary	50 times greater than iodide	0.2	43.1	31.0
5—Thyroid*	0	0.3	0.5	0
6—Thyroid	0	5.9	49.2	0
7—Thyroid	50 times greater than iodide	4.2	52.1	0

* Boiled homogenates.

iodide was used as substrate in Table 5, and it was also found that bromide fails to inhibit iodide utilization when 6.7×10^{-5} M iodide is employed. When 6.7×10^{-5} M KI^{131} is used as substrate in the absence of tyrosine, 6% of the radioactivity is found in the salivary protein, confirming an earlier report (9).

Studies on the Iodination of Histidine and Iodothyronines. The results in Table 6 demonstrate that histidine is iodinated by thyroid (incubation 2) and salivary (incubation 7) homogenates but tyrosine is overwhelmingly iodinated in an equimolar mixture of the two amino acids (incubations 3 and 8). Tyrosine is preferentially iodinated even though its concentration

TABLE 6. IODINATION OF HISTIDINE BY THYROID AND SALIVARY HOMOGENATES

Conditions: All incubations were for 1 hour with 3.3×10^{-3} M glucose, 125 μg glucose oxidase and the amino acid indicated in a concentration of 3.3×10^{-3} M except for incubation number 4 whose tyrosine concentration was 3.3×10^{-4} M. Incubations 1–5 had 0.5 ml. thyroid homogenate and 6.7×10^{-5} M KI^{131} in a total volume of 1.5 ml. while 6–8 had 1.0 ml. salivary homogenate and 3.3×10^{-4} M KI^{131} in a total volume of 3.0 ml.

Incubation number and homogenate*	Amino acid added	% of added iodide incorporated into:			
		Protein	MIT	DIT	MIH†
1—Thyroid	L-Tyrosine	6.9	58.9	0	0
2—Thyroid	L-Histidine	35.8	4.6	0	12.9
3—Thyroid	L-Histidine+L-Tyrosine	7.8	53.7	0.7	3.2
4—Thyroid	L-Histidine+L-Tyrosine	12.1	30.5	1.2	5.8
5—Thyroid	0	27.7	3.1	0.7	0.3
6—Salivary	L-Tyrosine	0.2	36.8	15.0	0
7—Salivary	L-Histidine	0.2	0	0	52.6
8—Salivary	L-Histidine+L-Tyrosine	0.2	31.3	12.3	8.8

* Boiled homogenates are inactive.
† MIH refers to monoiodohistidine.

is one-tenth as great as histidine (incubation 4). Analyses of incubations 2 and 7 showed that only monoiodohistidine (Rf = 0.18) contained radioiodide and that no diiodohistidine was formed by either salivary or thyroid glands. The MIT formed in incubations 2 and 5 is from iodination of endogenous tyrosine in the thyroid homogenate while no MIT was formed in incubation 7 because the salivary homogenate had been dialyzed.

The possible thyroxine precursors, thyronine, 3,5-diiodothyronine and 3,5,3′-triiodothyronine (10^{-5} M), are not iodinated by human or rat thyroid homogenates under conditions where iodide (10^{-5} M) is readily incorporated into tyrosine. In addition, radioiodide does not exchange with the iodine in thyroxine.

DISCUSSION

In thyroid glands the major mechanism for converting iodide to a reactive chemical species that can be organically bound is oxidation by a peroxidase. Support for this mechanism comes from the finding that other biological oxidants and electron carriers, except flavins, cannot replace H_2O_2 and from independent reports (2, 10) that H_2O_2 augmented iodide

metabolism. Flavin stimulation is abolished by catalase or anaerobiosis and is therefore due to the production of H_2O by the aerobic autoxidation of the flavin. These results have been verified in numerous repeat experiments and contradict the report by Tong *et al.* (3) that flavin stimulation is not affected by anaerobic (nitrogen atmosphere) conditions, although their data indicate that catalase inhibits flavin stimulation at pH 7.5. Recently, Soodak and Maloof (31) have verified the inhibition of flavin stimulation by catalase and anaerobiosis. The requirement for light (4) would be explained by the photochemical activation of flavins (11, 12).

Others (2, 13) have believed copper essential for iodide oxidation because Cu^{++} was required for MIT synthesis from *carrier-free* radioiodide. The requirement for added Cu^{++} may be explained by the inhibition of a TPNH dependent MIT and DIT deiodinase described by Stanbury (14, 15). He showed that the deiodinase is in thyroid and other tissues and that it is inhibited by copper. In the absence of Cu^{++}, the enzyme and limiting concentrations of endogenous TPNH in homogenates could deiodinate the small amount of MIT (less than 10^{-6} M) formed from carrier-free iodide, whereas only a negligible percentage of the synthesized iodotyrosines (10^{-3} to 10^{-5} M) would be deiodinated in the author's experiments. Taurog *et al.* (16) observed no requirement for added Cu^{++} because their preparations were producing protein bound MIT which is not dehalogenated. The *in vivo* role of Cu^{++} in iodine metabolism is problematical.

Hydrogen peroxide may be produced in thyroid glands by flavin enzymes since flavin adenine dinucleotide is a thyroid constituent (17). Data on the presence of such an enzyme, xanthine oxidase, in the glands are conflicting (17, 18). Peroxide may also be generated from the autoxidation of ubiquitous substances like sulfhydryl compounds and ascorbic acid. Some sporadic goiters are possibly due to a deficiency in the peroxide generating mechanism and indeed, absence of iodide peroxidase is the most likely biochemical defect of those congenital goitrous disorders (19) in which thyroid glands trap iodide but cannot metabolize it even when H_2O_2 is supplied (10).

After inorganic iodide is oxidized, the iodination of tyrosine is probably enzymatically controlled, as proposed by Serif and Kirkwood (2), and this is further indicated by the inability of thyroid homogenates to iodinate thyronine and iodothyronines. This latter result strengthens the presumption (20) that thyroxine is not formed by iodination of free thyronine precursors. However, the postulated iodinating enzyme, which may be iodide peroxidase or another protein, can iodinate histidine. This was anticipated because iodohistidine had been previously isolated from thyroid gland hydrolysates (21), and preferential iodination of tyrosine explains iodohistidine as a minor thyroid constituent.

When supplied with H_2O_2, milk (22, 23, 24, 25), lacrimal (9) and salivary glands can readily synthesize MIT, DIT and iodohistidine, but they can-

not store organically bound iodine or synthesize thyroxine. These biological materials contain peroxidases which, like thyroid iodide peroxidase, are completely inhibited by relatively small concentrations of some goitrogenic agents (1, 23, 26), but salivary and lacrimal peroxidases are markedly different from thyroid peroxidase in that they oxidize bromide as well as iodide.

Bromide[82] is not fixed into organic compounds of the thyroid *in vivo* (27, 28), consistent with the observation that these homogenates do not oxidize this halide. Although salivary homogenates readily peroxidize bromide, bromination of tyrosine does not proceed as well as iodination and the difference might be that iodine yields I^+ more easily than bromine gives rise to Br^+ (29, 30). Moreover, at neutral pH the chemical oxidation of bromide by H_2O_2 proceeds much slower than iodide oxidation. It is interesting that iodide utilization by salivary homogenates is unaffected by large concentrations of bromide. This may be due to a greater affinity of peroxidase for iodide or might be the result of oxidation of iodide by bromine that is formed by the enzyme.

Acknowledgment

The technical assistance of Miss Betty J. Corcoran is gratefully acknowledged.

REFERENCES

1. Alexander, N. M.: *J. Biol. Chem.* **234:** 1530. 1959.
2. Serif, G. S. and S. Kirkwood: *J. Biol. Chem.* **233:** 109. 1958.
3. Tong, W., A. Taurog and I. L. Chaikoff: *J. Biol. Chem.* **227:** 773. 1957.
4. Soodak, M. and F. Maloof: *Fed. Proc.* **18:** 329. 1959.
5. Roche, J., S. Lissitzky, O. Michel and R. Michel: *Ann. pharm. franç.* **9:** 163. 1951.
6. Yagi, Y., R. Michel and J. Roche: *Ann. pharm. franç.* **11:** 30. 1953.
7. Roche, J., S. Lissitzky and R. Michel: Methods of Biochemical Analyses, New York, Interscience Publishers Inc., vol. I, pp. 243–264. 1954.
8. Ingold, C. K.: Structure and mechanisms in organic chemistry, Ithaca, 290. 1953.
9. Serif, G. S. and S. Kirkwood: Trace Metals, N. Y., Academic Press, Inc., pp. 255–267. 1958.
10. Haddad, H. M. and J. B. Sidbury, Jr.: *J. Clin. Endocrinol. and Metab.* **19:** 1446. 1959.
11. Frisell, W. R., C. W. Chung and C. G. Mackenzie: *J. Biol. Chem.* **234:** 1297. 1959.
12. Haliwer, M.: *J. Am. Chem. Soc.* **73:** 4870. 1951.
13. Weiss, B.: *J. Biol. Chem.* **201:** 31. 1953.
14. Stanbury, J. B.: *J. Biol. Chem.* **228:** 801. 1957.
15. Stanbury, J. B.: *J. Biol. Chem.* **233:** 106. 1958.
16. Taurog, A., G. D. Potter, and I. L. Chaikoff: *J. Biol. Chem.* **213:** 119. 1955.
17. Pihar, O.: *Časopis Lékařů Českých* **91:** 833. 1952; *Chem. Abstracts* **49:** 7089f. 1955.
18. Schein, A. H.: *Endocrinology* **59:** 596. 1956.
19. Stanbury, J. B. and E. M. McGirr: *Am. J. Med.* **22:** 712. 1957.
20. Roche, J. and R. Michel: *Adv. in Protein Chem.* VI: 258. 1951.

21. Roche, J., S. Lissitzky and R. Michel: *Biochim. et Biophys. Acta* **8:** 339. 1952.
22. Keston, A. S.: *J. Biol. Chem.* **153:** 335. 1944.
23. Pitney, W. R. and T. R. Fraser: *J. Endocrinol.* **9:** 224. 1953.
24. Roche, J., R. Michel and E. Volpert: *Compt. rend. Soc. biol.* **148:** 21. 1954.
25. MacQuillan, M. T., R. K. Morton, P. G. Stanley and V. M. Trikojus: *Nature* **173:** 305. 1954.
26. Alexander, N. M.: *J. Biol. Chem.* **234:** 148. 1959.
27. Yagi, Y., R. Michel and J. Roche: *Bull. soc. chim. biol.* **35:** 289. 1953.
28. Perlman, I., M. E. Morton and I. L. Chaikoff: *Am. J. Physiol.* **134:** 107. 1941.
29. Murray, H. D.: *J. Chem. Soc.* **127:** 882. 1925.
30. Berliner, E.: *J. Am. Chem. Soc.* **72:** 4003. 1950.
31. Soodak, M. and F. Maloof: *Fed. Proc.* **19:** 173. 1960.

FURTHER STUDIES ON PROGESTIN BIOASSAY USING THE ENDOMETRIAL RESPONSE IN THE RABBIT[1]

YASUNAO OGAWA AND GREGORY PINCUS

The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

ABSTRACT

Using a micromanometric method for the determination of carbonic anhydrase, a comparative study has been made of the dosage response relationship and the concentration of this enzyme with the increase in endometrial weight in Clauberg-type rabbit uteri following progesterone administration. A linear relationship to log. dose has been found for carbonic anhydrase concentration over the range 0.125 to 2 mg. of progesterone, and over the range 0.25 to 4 mg. for endometrial weight. Similar linear dose response curves have been found with the synthetic progestin, the enol propionate of 19-nor 17α-ethyl testosterone (SC-7294). Its potency ratio to progesterone is approximately 2. Following a single injection of either progesterone or SC-7294, the time course of change in endometrial weight and enzyme concentration has been followed over an 8- to 10-day period. An increase in endometrial weight precedes the increase in carbonic anhydrase activity. Following a single injection of progesterone to immature, unprimed female rabbits examined on the fourth day after administration, a linear log. dose response curve is obtained over the range 0.6 to 16 mg. for carbonic anhydrase concentration. In this single injection assay the endometrial weight response is much more limited and highly variable.

PREVIOUS studies have shown that the quantitative estimation by carbonic anhydrase concentration of the progestational activity in the rabbit endometrium correlates well with the degree of pseudopregnant proliferation (1–4). It was also demonstrated that the Lutwak-Mann test for progestin-induced carbonic anhydrase increase may be used as a quantitative bioassay for progestational and antiprogestational effects (2, 3). A manometric micro-method for the determination of carbonic anhydrase activity, which was recently reported as a sensitive, accurate and practical method suitable for biological studies (5), would make this bioassay method more useful.

In this paper we present: (a) statistical analysis of the dose-response relationships between progesterone and endometrial carbonic anhydrase concentration and also between progesterone and endometrial weight in the Clauberg rabbit, (b) a comparative assay of progesterone and another

Received September 19, 1960.

[1] Investigations described in this paper have been carried on under Contract No. SA-43-ph-3029 from the Cancer Chemotherapy National Service Center, National Cancer Institute, Public Health Service.

progestin, the 3-propionate of 17α-ethyl-19-nortestosterone (SC-7294),[2] (c) the time course of change in endometrial carbonic anhydrase concentration and in endometrial weight after a single administration of progestin, and (d) a simplified assay using a single injection of progesterone.

METHODS

Immature albino rabbits (1.3±0.2 kg.) were used. To prime the rabbits, 5 μg of estradiol was injected subcutaneously once daily for 6 days. Administration of progestin was begun on the 7th day and continued daily for 5 days. Twenty-four hours after the last injection the rabbits were sacrificed and the endometria were scraped off with a dull surgical knife.

The preparation of the enzyme extract and the procedures for the determination of enzyme activity were the same as those described in our previous paper (5). All hormones were dissolved in Sesame Oil U.S.P. and the volume of injection was 0.2 ml. per rabbit.

RESULTS

Bioassay of Progesterone by a Multiple Administration in the Clauberg Rabbits

The data on the carbonic anhydrase concentration and the amount of endometrium in a series of control and progesterone-treated animals are shown in Table 1 and illustrated in Figure 1. Statistical analysis gave

TABLE 1. THE PROGESTERONE DOSE-RESPONSE RELATIONSHIPS FOR CARBONIC ANHYDRASE CONCENTRATION AND ENDOMETRIAL WEIGHT OF CLAUBERG RABBITS

Dosage* (mg./rabbit)	No. of rabbits	Endometrial weight (mg.)	Carbonic anhydrase concentration (E.U./gm.)
		Mean ± S.E.	Mean ± S.E.
0	10	38.8 ± 5.28	157.8 ± 17.69
0.125	10	40.3 ± 4.53	314.1 ± 27.99
0.25	10	89.4 ± 11.71	476.2 ± 33.81
0.5	10	380.5 ± 48.74	726.2 ± 48.77
1.0	10	592.7 ± 67.26	952.6 ± 57.48
2.0	10	813.4 ± 59.83	1078.5 ± 71.61
4.0	8	1121.0 ± 177.89	1130.3 ± 77.93
8.0	8	1162.7 ± 114.05	1132.0 ± 109.36

* Dosage of progesterone shows the total amount administered in five consecutive days.

evidence of the linear logarithmic dose response relationships over the dosage range of 0.125 to 2 mg. for the endometrial carbonic anhydrase concentration, and over the range of 0.25 to 4 mg. for the endometrial weight (Tables 2 and 3).

At the usable upper-limit dosages for these dose-response regression lines, the endometrial weight shows a nearly 30-fold increase over the control level, while the enzyme concentrations show about an 8-fold increase. However, the larger value of the precision index for the endometrial weight measurements ($\lambda = 0.305$), compared to the enzyme con-

[2] The progestin SC-7294 was supplied to us by Dr. A. L. Raymond of G. D. Searle & Co.

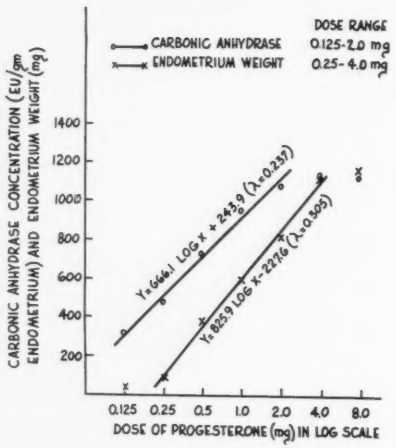

FIG. 1. Dose response regression line of progesterone (Clauberg rabbits).

centration measurements ($\lambda = 0.237$), indicates the larger individual difference in the responses to progesterone. Furthermore, the endometrial carbonic anhydrase concentration is sensitive enough to the dose of progesterone as low as 0.125 mg. per rabbit. These facts point to the accuracy

TABLE 2. STATISTICAL ANALYSIS OF LINEAR RELATIONSHIP BETWEEN PROGESTERONE DOSE AND CARBONIC ANHYDRASE CONCENTRATION

(Dosage range of progesterone: 0.125–2.0 mg./rabbit)

Factor	Sum of sq.	d.f.	Variance
Linearity		1	4,020,598.51
Residue	42,217.63	3	14,072.54
Dose	4,062,816.13	(4)	
Error	1,130,752.36	45	25,127.82
Total	5,193,568.49	49	

$F = 0.56 < F_{43}^{3} = 1.54$ at $P = 0.2$; $F' = 160.0 \gg F_{43}^{1} = 12.45$ at $P = 0.001$.
Linear regression is highly significant.
$\gamma = 661.1$ Log. $X + 243.9$; $\lambda = 0.237$.

TABLE 3. STATISTICAL ANALYSIS OF LINEAR RELATIONSHIP BETWEEN PROGESTERONE DOSE AND ENDOMETRIAL WEIGHT

(Dosage range of progesterone: 0.25–4.0 mg./rabbit)

Factor	Sum of sq.	d.f.	Variance
Linearity		1	5,665,410.20
Residue	27,031.12	3	9,010.37
Dose	5,692,441.32	(4)	
Error	2,727,555.42	43	63,431.52
Total	8,419,996.74	47	

$F = 0.142 < F_{43}^{3} = 1.55$ at $P = 0.2$; $F' = 89.32 \gg F_{43}^{1} = 12.51$ at $P = 0.001$.
Linear regression is highly significant.
$\gamma = 825.9$ Log. $X - 227.6$; $\lambda = 0.305$.

and usefulness of this enzyme method for the quantitative assay of progestin.

Comparative Assay of Progesterone and SC-7294

Table 4 shows the data on a comparative assay of a progestational compound, SC-7294, against progesterone using measurements of endometrial carbonic anhydrase concentration and the endometrial weight in Clauberg rabbits. Both types of measurements gave entirely parallel regression

TABLE 4. COMPARATIVE ASSAY OF PROGESTERONE AND SC-7294 BY ENDOMETRIAL CARBONIC ANHYDRASE CONCENTRATION AND ENDOMETRIAL WEIGHT OF CLAUBERG RABBITS

Dosage* (mg./rabbit)	No. of rabbits	Endometrial weight (mg.)	Carbonic anhydrase concentration (E.U./gm.)
		Mean ± S.E.	Mean ± S.E.
		Experimental Data	
Progesterone			
0.125	6	44.2 ± 6.18	309.6 ± 44.32
0.5	6	221.7 ± 49.31	710.7 ± 28.27
2.0	6	764.2 ± 61.22	1080.8 ± 74.64
SC-7294			
0.0625	6	58.2 ± 16.45	348.5 ± 28.74
0.25	6	316.7 ± 67.32	616.3 ± 25.19
1.0	6	845.0 ± 32.81	1085.9 ± 51.67
		Analytical Data	
Method		4-Point assay†	6-Point assay
Parallelism (P = 0.05)		Highly significant	Highly significant
Regression coefficient		267.72	377.12
Logarithm of potency ratio		0.3998	0.2742
Potency ratio = $\frac{\text{SC-7294}}{\text{Progesterone}}$		2.511	1.880
Fiducial limit (P = 0.05)		52.7—213.2%	71.1—140.8%

* Dosage shows the total amount administered in five consecutive days.
† Data of the lowest dosage are discarded.

lines, although there was some difference in the potency ratios. In the analysis of the endometrial weight assay, however, the data of the lowest dosages, which are too small for the endometrial weight response, were discarded and a 4-point assay about the middle and high dosages was carried out, instead of a 6-point assay which is the most reliable method of a comparative assay.

SC-7294 is estimated as a progestin about two times as active as progesterone.

Time Course of Carbonic Anhydrase Concentration and Endometrial Weight after a Single Administration of Progesterone and SC-7294

Using Clauberg rabbits, time studies on variations in carbonic anhydrase concentration and endometrial weight were pursued after a single injec-

tion of progesterone (4 mg. per rabbit) or SC-7294 (3 mg. per rabbit). The data in Table 5 show that both compounds caused a remarkable increase of the endometrial weight which was followed by a rather rapid decrease after an early peak, but that the enzyme concentration increased rather slowly, and reached its peak 2 or 3 days later than endometrial weight. It is clear that SC-7294 also possesses a greater activity in a single injection, as well as in the standard assay, and that it has a longer lasting activity than progesterone using both types of measurements.

TABLE 5. VARIANCE OF CARBONIC ANHYDRASE CONCENTRATION AND ENDOMETRIAL WEIGHT IN THE TIME COURSE AFTER A SINGLE INJECTION OF PROGESTINS

(Clauberg rabbits)

Time course after injection (day)	No. of rabbits	Endometrial weight (mg.)	Carbonic anhydrase concentration (E.U./gm.)
		Mean ±S.E.	Mean ±S.E.
Progesterone: 4.0 mg.			
1	6	103.5± 17.92	408.8± 34.91
2	6	706.3± 94.73	719.3± 26.84
3	6	485.2± 55.36	995.6± 72.14
4	6	420.7±101.66	1376.3± 71.97
5	6	317.8± 99.38	1773.9± 71.05
6	6	70.2± 17.73	1107.4± 42.86
7	6	24.2± 5.35	631.8± 83.63
8	6	23.0± 1.37	538.6± 65.10
SC-7294: 3.0 mg.			
2	5	608.0±109.18	361.6± 22.82
4	5	845.8±279.63	906.2± 67.16
6	5	265.8±122.35	2103.2±203.97
8	5	36.2± 9.89	780.3± 74.05
10	4	22.1± 6.11	480.6± 42.65

Bioassay of Progesterone by a Single Administration in Non-primed Immature Rabbits

Comparing the response caused by five consecutive days' injections in the total amount of 4 mg. progesterone (Table 1) with the response to a single injection of the same dosage (Table 5), it is noticeable that at the fifth or sixth day after the injection a higher carbonic anhydrase concentration resulted from a single injection, whereas a multiple administration causes a greater increase in endometrial weight. Taking notice of this fact, a bioassay method by a single injection was examined. In this trial, attempting to simplify the assay procedure, non-primed immature rabbits weighing about 1.7 kg. were used and sacrificed four days after the injection. As shown in Table 6 and Fig. 2, linear logarithmic dose response relationships were obtained over the dosage range of 0.5 to 16 mg. for the endometrial enzyme concentration and over the range of 1.0 to 16 mg. for the endometrial weight. The precision index value for the enzyme concentration measurements indicates a satisfactory degree of accuracy ($\lambda = 0.184$ and the linear relationship spreads over a wide range of progesterone, although the minimum detectable dosage is approximately 0.5 mg. On the

TABLE 6. THE PROGESTERONE DOSE-RESPONSE RELATIONSHIPS FOR CARBONIC ANHYDRASE CONCENTRATION AND ENDOMETRIAL WEIGHT OF NON-PRIMED IMMATURE RABBITS

(Four days after single dose administration of progesterone)

Dosage (mg./rabbit)	No. of rabbits	Endometrial weight (mg.)	Carbonic anhydrase concentration (E.U./gm.)
		Mean ± S.E.	Mean ± S.E.
		Experimental Data	
0.5	8	26.0 ± 2.52	271.5 ± 19.26
1.0	8	67.4 ± 13.26	430.5 ± 23.47
2.0	8	107.8 ± 11.71	715.6 ± 58.50
4.0	8	200.1 ± 43.28	978.2 ± 60.44
8.0	8	311.6 ± 50.24	1291.6 ± 63.32
16.0	8	385.3 ± 68.82	1496.0 ± 80.16
		Analytical Data	
Dosage range		1.0–16.0 mg.	0.5–16.0 mg.
Variance: Linearity		564,118.24	9,192,845.60
Residue		3,548.11	14,499.95
Error		15,105.62	24,534.86
Linear regression (P = 0.001)		Highly significant	Highly significant
Regression line		$\gamma = 278.9$ Log. X − 232.5	$\gamma = 851.2$ Log. X − 371.6
Precision index (λ)		0.441	0.184

other hand, as might have been expected as the result of using non-primed rabbits both the response and the accuracy of the endometrial weight measurements are unsatisfactory.

DISCUSSION

In previous papers (2, 3) it was confirmed that the increase of the endometrial carbonic anhydrase in the Clauberg rabbits is usable as a bioassay method for progestational and antiprogestational activity.

As the result of our use of a manometric micro-determination of the

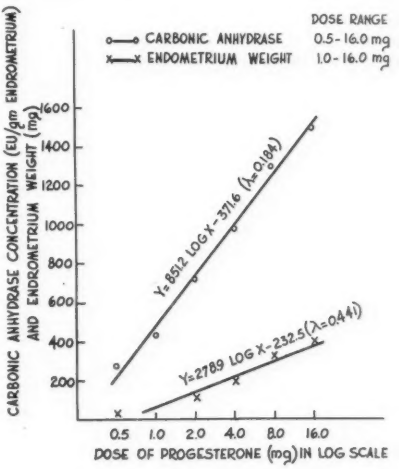

FIG. 2. Dose response regression line of progesterone (non-primed rabbits).

enzyme activity, the sensitivity of the dose response relationship has been increased so that the minimum detectable dosage of progesterone comes down to 0.125 mg., and the maximum response is obtained at 2.0 mg. per rabbit. Furthermore, a 6-point comparative assay of a progestational compound, SC-7294, using progesterone as a standard, showed entirely parallel regression lines, indicating the accuracy and reliability of the carbonic anhydrase method using multiple injections in the Clauberg rabbits as a standard assay procedure. Finally, as a simpler method, the carbonic anhydrase assay by a single injection in the non-primed rabbits has been found to have a satisfactory degree of accuracy and a rather wide range to varying progesterone dosage, in which the minimum detectable dosage is approximately 0.5 mg. per rabbit, the same as in the previously reported non-manometric methods (2, 6).

On the other hand, when the endometrial weight was used as an indicator of the endometrial proliferation, it showed statistically linear dose response relationships in all experiments, but a higher dosage range was required than that for the carbonic anhydrase response. However, these endometrial weight responses were accompanied by a wider range of standard error and seemed to lack the necessary degree of accuracy needed for a quantitative assay.

In the experimental data on variations of carbonic anhydrase concentration and of the endometrial weight after a single injection of progestins, it was observed that the maximum response of the endometrial weight appeared before that of the enzyme concentration. The two injection schedules could account for the difference in the increase of the enzyme and the increase of the tissue weight. Also these responses would suggest a possible connection with the mechanism of the carbonic anhydrase biosynthesis by progestins in the rabbit endometrium i.e. tissue synthesis preceding enzyme activity increase.

In the routine search for new progestational compounds, it is most desirable to have a simple and reliable assay method. This paper presents a series of working procedures suitable for that purpose, that is: an assay procedure by a multiple injection of progestin in the Clauberg rabbit with (1) screening by checking the endometrial weight increase, (2) estimation of the progestational activity by the measurements of the carbonic anhydrase concentration, and then, if necessary (3) the estimation of the potency ratio by a comparative assay.

REFERENCES

1. Lutwak-Mann, C. and C. E. Adams: *J. Endocrinol.* **15**: 42. 1957.
2. Pincus, G., T. Miyake, A. P. Merrill and P. Longo: *Endocrinology* **61**: 528. 1957.
3. Miyake, T. and G. Pincus: *Proc. Soc. Exper. Biol. & Med.* **99**: 478. 1958.
4. Miyake, T. and G. Pincus: *Endocrinology* **63**: 816. 1958.
5. Ogawa, Y. and G. Pincus: *Endocrinology* **67**: 551. 1960.
6. Pincus, G. and N. T. Werthessen: *Am. J. Physiol.* **120**: 100. 1937.

THE INDUCTION OF OVULATION IN STARVING PULLETS USING MAMMALIAN AND AVIAN GONADOTROPINS[1]

T. R. MORRIS[2] AND A. V. NALBANDOV

Department of Animal Science, University of Illinois, Urbana, Illinois

ABSTRACT

Laying pullets deprived of all nourishment, except water and oyster shell grit, showed ovarian degeneration and cessation of ovulation within 72 hours. Injections of gonadotropins prevented ovarian degeneration and, in some cases, induced regular daily ovulations for 8 or more days after removal of food. With the limited range of doses investigated, intramuscular injections of dried chicken anterior pituitary glands were more effective in causing daily ovulations than was a combination of intramuscular injections of mammalian follicle stimulating hormone with intravenous injections of mammalian luteinizing hormone. There was a progressive decline in the weight of yolks ovulated by starved, hormone-supported pullets.

It is suggested that failure to lay in the starved pullet is primarily due to failure of the pituitary to secrete adequate gonadotropins. The possibility that less extreme dietary deficiencies affect egg production via pituitary function is discussed briefly.

IT WAS shown by Hosoda and his associates (1, 2) that when a laying hen is starved there is normally a rapid degeneration of the ovary, but that this can be prevented by injecting mammalian gonadotropins. In one experiment ovulations were induced by a single intravenous injection of luteinizing hormone (LH) given at the end of a 7-day starvation period, during which the ovary had been supported by daily injections of follicle stimulating hormone (FSH) or pregnant mare serum (PMS).

In Hosoda's experiments the principal effect of hormone treatment was to maintain the ovary in the condition prevailing at the beginning of the starvation period. Although ovarian weight was considerably above normal for some of the starved, gonadotropin-treated hens, indicating the deposition of some yolk during starvation, no attempt was made to maintain a normal rate of yolk synthesis or to induce repeated ovulations.

The series of experiments reported below was undertaken to determine to what extent laying pullets could be induced to continue yolk synthesis during a period of starvation, with daily removal of yolk material by induced ovulation.

Received September 24, 1960.

[1] This work was conducted during the senior author's tenure of a Post Doctoral Fellowship at the University of Illinois.

[2] Present address: Department of Agriculture, University of Reading, England.

MATERIALS AND METHODS

White Leghorn pullets between 6 and 10 months of age were kept in individual cages. Artificial lights were used from 6 A.M. to 8 P.M. The diet for all birds before each experiment, and for fed controls during each experiment, was a pelleted laying ration (21% protein) and mixed grain given at the discretion of the attendant. "Starved" pullets were deprived of all food (except oyster shell) throughout each experiment. Feed was removed at 5 P.M. in Experiments 1 and 2, at 12 Noon in Experiment 3 and at 10 A.M. in Experiment 4. Starving birds had unlimited access to water and in the last two experiments they also had a supply of oyster shell grit. Subsidiary trials indicated that the provision of this calcium source did not affect ovulation rate but did substantially reduce the proportion of soft-shelled eggs. Soft-shelled eggs were likely either to be eaten or to fall through the cage floor and break.

The mammalian hormones used were commercial preparations of FSH (Armour Lot No. R 377201) and LH (Armour Lot No. R 377279). Assay of these hormone preparations on rats showed that neither preparation was pure and can at best be characterized as "predominantly FSH or LH." According to the manufacturer the FSH contained about 5% LH on a dry matter basis, while the LH contained "some" prolactin and thyrotrophin. The LH-contaminant in FSH was sufficient either to maintain comb size in hypophysectomized hens or, if given in sufficiently high doses, actually to cause comb growth (3). Evidence to be presented later (manuscripts in preparation of Opel's data (3) on the maintenance of ovarian function in hypophysectomized hens), will show that the LH preparation also had a significant amount of FSH which appeared to be negligible when assayed by accepted methods on rats.

Chicken pituitary powder (CAP) was made by lyophilizing anterior pituitary glands obtained from 8- to 12-week old chickens of both sexes. This preparation was crude and contained all the hypophyseal hormones usually expected in such powder. The dried CAP powder was suspended in saline at a concentration of 2.5 mg. or 10.0 mg. per ml. The crudeness of this material accounts for the large doses of the powder which had to be injected in order to obtain the desired degrees of stimulation.

In Experiment 2, starved controls were injected with saline and received the same handling as birds on other treatments. In later experiments the starved controls were not injected.

In each experiment, the day on which food was removed was designated "Day 0" and injections were begun on the following day, "Day 1." In Experiment 2 injections were made daily between 10 P.M. and 11 P.M. and in Experiments 3 and 4 injections were made daily between 11 A.M. and noon. Initial injections were made 23–28 hours after removal of food in each experiment.

Records of lay were available for at least a week prior to the start of each trial. These were used to ensure that all birds had been laying regularly, but no effort was made to match treatment groups. Birds were palpated at the time of each injection for the presence of an egg in the oviduct. In some cases eggs were not laid within 24 hours of being first palpated and efforts were then made to induce oviposition with an intravenous injection of 2–12 U.S.P. units of Purified Oxytocic Principle. These injections induced oviposition in approximately half the cases and had no apparent effect on subsequent events in the ovary. Eggs which were held in the uterus beyond 24 hours were recorded as though they had been laid when they were due. Eggs found in the uterus at autopsy (other than those held over) were recorded as being laid on the day of autopsy.

RESULTS

Experiment 1. Rate of Ovarian Regression

The first experiment was designed to determine how rapidly ovarian regression sets in following removal of food from young pullets. A group

of 26 pullets was divided into two lots. One lot was fed normally while the other lot was starved until egg production had ceased (on Day 5) at which time 4 birds were autopsied. A separate group of 12 pullets was starved and lots of 3 birds were sacrificed at intervals of 24, 48, 72 and 96 hours after removal of food. The results are presented in Table 1.

The first signs of follicular atresia were apparent 48 hours after removal of food. By the afternoon of day 3 the majority of follicles, including all those of normally ovulable size, were atretic.

TABLE 1. RESULTS OF SIMPLE STARVATION

Treatment		No. of pullets	Per cent egg production*								
			Day −3	Day −2	Day −1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
1. Fed		13	92	69	85	62	62	69	77	92	69
2. Starved (from day 0)		13	92	85	92	85	85	85	54	8	0
Autopsy data for starved pullets	No. of birds autopsied						3	3	3	3	4
	Mean ovary weight (gm.)						58.9	46.9	24.6	30.4	15.3
	No. of normal follicles (<1 gm.) per ovary						6.3	6.0	1.0	1.7	0
	Mean oviduct weight (gm.)						59.3	51.0	37.6	36.8	15.8

* Per cent production $= \frac{\text{No. of eggs} \times 100}{\text{No. of birds} \times \text{No. of days}}$.

Since food was removed late in the afternoon of day 0, assimilation of nutrients from the gut presumably continued for some time into the morning of Day 1. On Day 2, or about 24 hours after the onset of real starvation, almost half the birds failed to ovulate (ovipositions recorded on Day 3 result from ovulations on Day 2). Only one bird ovulated on Day 3 and she had failed to ovulate on Day 2.

Thus starvation first affected events in the ovary about 24 hours after the gut became empty; after 48 hours the ovaries of most pullets were atretic and devoid of ovulable follicles.

Experiment 2. Ovarian support and ovulation with mammalian gonadotropins

In this trial FSH was used to support ovarian growth, as indicated by the experiments of Hosoda *et al.* (1, 2). In addition, daily intravenous injections of LH were given in an attempt to induce daily ovulations. Nine daily FSH injections were made beginning on Day 1 and eight LH injections were made, beginning on Day 2. All birds were autopsied in the afternoon of Day 10. Other details of treatments and a summary of results are presented in Table 2.

FSH alone was effective in maintaining ovarian weight and resulted in some ovulations beyond the fifth day, when the starved controls laid their last eggs. Intravenous injections of LH, in addition to causing some ovulations, were about as effective as the intramuscular injections of FSH in supporting ovarian weight. FSH and LH together gave a slightly higher

TABLE 2. RESULTS OF INJECTING MAMMALIAN GONADOTROPINS INTO STARVING PULLETS

Treatment (per bird per day)	No. of pullets	Per cent egg production*				Autopsy data (Day 10)	
		Days −3–0	Days 1–4	Days 5–8	Days 9–10	Ovary wt. (gm.) mean ± s.e.	Oviduct wt. (gm.) mean ± s.e.
1. Fed controls	5	90	90	90	90	48.2 ± 3.8	60.4 ± 8.2
2. Starved controls	5	80	65	15	0	9.3 ± 1.1	12.7 ± 1.8
3. 0.5 units FSH, i.m.	5	85	70	40	10	60.8 ± 10.4	62.3 ± 10.1
4. 1 mg. LH, i.v.	5	95	75	10	30	62.0 ± 3.6	53.5 ± 2.4
5. 0.5 units FSH, i.m. + 1 mg. LH, i.v.	10	85	70	40	20	85.8 ± 9.1	53.4 ± 4.8

* Per cent production = $\frac{\text{Number of eggs} \times 100}{\text{No. of birds} \times \text{No. of days}}$.

i.m. = intramuscular; i.v. = intravenous.

ovulation rate than either one alone, but differences between the three hormone-treated groups were not significant. The greater total dose of gonadotropins received by birds on treatment 5 is reflected in their significantly greater ovarian weights ($P < 0.05$ when compared to treatments 3 and 4). Three of the pullets on treatment 3 died on Day 10.

At autopsy, most of the ovaries from the gonadotropin-treated birds showed an excess of small (1–4 gm.) and medium (5–10 gm.) follicles with a lack of large (11–16 gm.) follicles and a complete absence of the follicular hierarchy which characterizes the ovary of a normal pullet. The proliferation of small and medium sized follicles was particularly noticeable with treatment 5 (see Fig. 1 C). Most of the ovaries showed one or two atretic follicles at autopsy.

This experiment demonstrated that it was possible to obtain more than one ovulation from a starving pullet and indeed 9 (out of 20) hormone-treated birds each produced 5 or more eggs after the removal of food. However, the average yield of the hormone-treated pullets was only 3.65 eggs per bird for Days 2 through 10, compared with 2.4 eggs per bird for starved controls.

Experiments 3 and 4. Avian Gonadotropins

Recent experiments in this laboratory had shown that a crude chicken pituitary preparation was very effective in maintaining follicular growth and ovulation in hypophysectomized pullets (3). There was some indication that this material was more effective in maintaining a nearly normal follicular hierarchy than were the usual mammalian gonadotropins and so the material was tested in starving birds.

Two experiments were conducted with identical materials and the results are presented jointly in Table 3. In an attempt to alleviate the technical difficulty of making repeated intravenous injections, the birds used for treatments 3 and 4 of Experiment 3 each had a polyethylene cannula

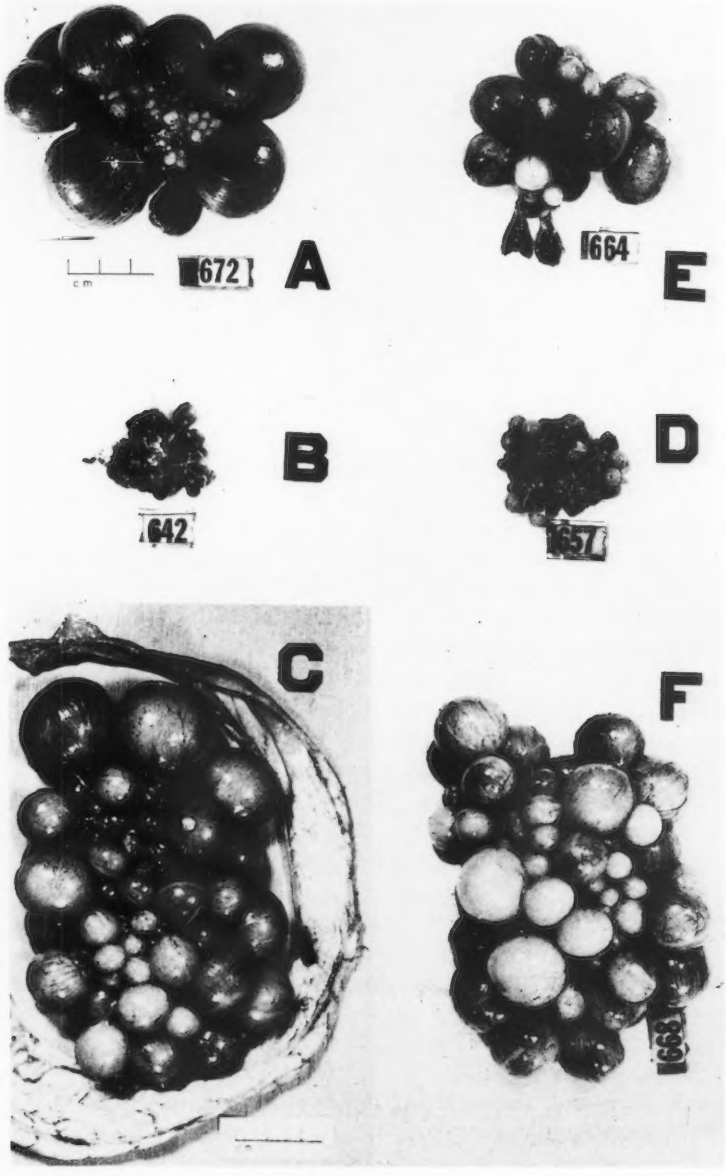

FIG. 1. Ovaries taken from pullets at autopsy. A—normal ovary from fed control. B—ovary from pullet starved for 11 days. C—ovary from pullet starved for 10 days and treated with FSH and LH (treatment 5, Experiment 3). D, E and F ovaries from pullets starved for 11 days and treated with 2 mg., 4 mg. and 8 mg. respectively of chicken anterior pituitary powder daily (treatments 3, 4 and 5 of Experiment 4).

TABLE 3. RESULTS OF INJECTING AVIAN PITUITARY POWDER INTO STARVING PULLETS

Treatment (per bird per day)	No. of pullets	Per cent egg production				Ovary wt. (gm.) mean ± s.e.	oviduct wt. (gm.) mean ± s.e.	Body weight loss (gm.) mean ± s.e.
		Days −3–0	Days 1–4	Days 5–8	Days 9–12			
Experiment 3								
1. Fed controls	10	80	75	93	80	(48.2)*	(60.4)*	—
2. Starved controls	10	83	55	0	0	7.0± 1.8	8.4±0.8	348± 18
3. 1 mg. CAP, i.m. 0.5 mg. CAP, i.v.	4	81	88	19	0	6.6± 1.2	21.1±3.6	422± 46
4. 2 mg. CAP, i.m. 0.5 mg. CAP, i.v.	4	75	81	38	25	24.2±11.3	36.5±2.1	455± 18
Experiment 4					(days 9–11)			
1. Fed controls	30	90	92	89	90	(48.2)*	(60.4)*	—
2. Starved controls	2	88	50	0	0	7.0± 1.0	10.6±0.5	395±125
3. 2 mg. CAP, i.m.	5	100	95	80	20	8.7± 3.5	30.8±1.0	700± 47
4. 4 mg. CAP, i.m.	5	90	105	80	33	44.3± 5.7	52.7±4.0	734± 26
5. 8 mg. CAP, i.m.	5	95	100	55	17	88.5±22.1	61.8±7.6	730± 57
6. 2 mg. CAP, i.m. 0.25 units FSH, i.m.	5	90	100	50	13	75.0±10.4	43.7±2.2	580± 36
7. 2 mg. CAP, i.m. 0.5 units FSH, i.m.	5	90	110	40	13	60.2± 8.5	48.7±3.7	598± 46
8. 0.5 units FSH, i.m.	3	92	100	42	0	69.4±16.2	49.7±4.2	670± 90

* Data from Experiment 2.
CAP = Chicken anterior pituitary powder; i.m. = intramuscular; i.v. = intravenous.

sutured into the right wing vein. The end of this tube was sealed with hot forceps after each injection, and before each injection a small piece was snipped off to allow the insertion of a blunt needle. These cannulae were successful for a period of 3–11 days after which the portion of the vein which carried the sutures would become necrotic and the bird would peck out the cannula. When a cannula failed, intravenous injections were discontinued for that bird. It was then noticed that several pullets continued to ovulate in response to a single daily intramuscular injection of CAP and so, in Experiment 4, only intramuscular injections were used.

Injections were made on Days 1 through 11 in Experiment 3 with autopsies on the morning of Day 12. In Experiment 4 injections were made for 10 days, beginning on Day 1, and birds were sacrificed in the afternoon of Day 11.

Experiment 3 indicated that CAP injections induced some ovulations but, with the highest dose used, there was a marked deficiency of ripening follicles in the ovary at autopsy. Since the lack of small follicles was in contrast to the results of FSH treatment it was decided to test, in Experiment 4, the effects of (a) raising the dose of CAP and (b) combining CAP with FSH injections.

Table 3 shows that the most successful procedure was to raise the dose of CAP. The optimum dose, as judged by number of eggs laid and condition of the ovaries at autopsy, was 4 mg. of CAP per bird per day. Ovulation rate was almost as good using 2 mg. of CAP per day, but at autopsy four out of five ovaries contained no yellow yolks (see Fig. 1 D). The ovaries from birds treated with 4 mg. CAP showed many follicles in the 2–6 gm. range with a deficiency of larger follicles (see Fig. 1 E). It should be noted,

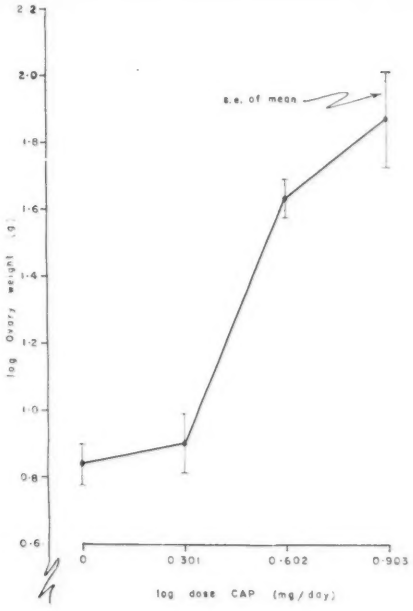

FIG. 2. Mean ovarian weight at autopsy for starving pullets injected with chicken anterior pituitary powder.

however, that the average weight of the follicles which ovulated on Days 9–10 was only 7 gm. (see below).

Birds receiving 8 mg. of CAP per day gave fewer ovulations than those given 2 mg. or 4 mg. At autopsy their ovaries exhibited the same pattern of overstimulation (a great excess of small (1–6 gm.) follicles) that is commonly seen in pullets treated with mammalian hormones (see Fig. 1 F).

Figure 2 shows the relation of ovarian weight at autopsy to dose of CAP. In all these experiments there was a marked tendency for the variance of both ovarian weights and oviduct weights to be proportional to the mean. This tendency could be corrected by logarithmic transformation.

The use of 0.25 or 0.5 units FSH in conjunction with 2 mg. CAP per day resulted in increased ovarian weights at autopsy but reduced rather than enhanced ovulation rate. The ovaries of birds receiving both FSH and CAP showed an excessive number of small follicles, similar in number and appearance to those obtained with the 8 mg. dose of CAP, but in many cases there was also extensive atresia of the larger follicles. The average number of atretic follicles per ovary was 5.8 for treatments 6 and 7 compared with 1.4 for treatments 3–5.

Treatment 8 was included as a check on the effect of injecting FSH alone. The results were similar to those obtained with this same treatment in Experiment 2.

Table 4 and Figure 3 summarize the daily performance of the ten pullets

TABLE 4. PERFORMANCE OF THE TEN BEST PULLETS IN EXPERIMENT 4*

Performance, days 1–11	Mean ±s.e.	Range
Eggs per bird	8.3± 0.33	7 – 11
Weight of eggs per bird (gm.)	404 ± 9.2	366 –464
Weight of yolk ovulated per bird (gm.)	108 ± 4.1	92 –125
Body weight loss (gm.)	745 ±25	640 –870
Ovary wt. at autopsy (gm.)	35.8± 8.6	5.0– 87.4

* 4 birds from treatment 3; 4 birds from treatment 4; 1 bird from treatment 5; 1 bird from treatment 6.

which gave the highest individual yields of egg material during starvation. It will be seen from Figure 3 that, in most of these cases regular daily ovulations were induced for about 7 days (about half the eggs laid on Day 2 resulted from induced ovulations, as judged by time of oviposition). Although there is no clutch pattern of the kind seen in normal chickens, it is possible for a bird to miss ovulation for one or two days and then to resume laying, presumably when the largest available follicle reaches "ovulable

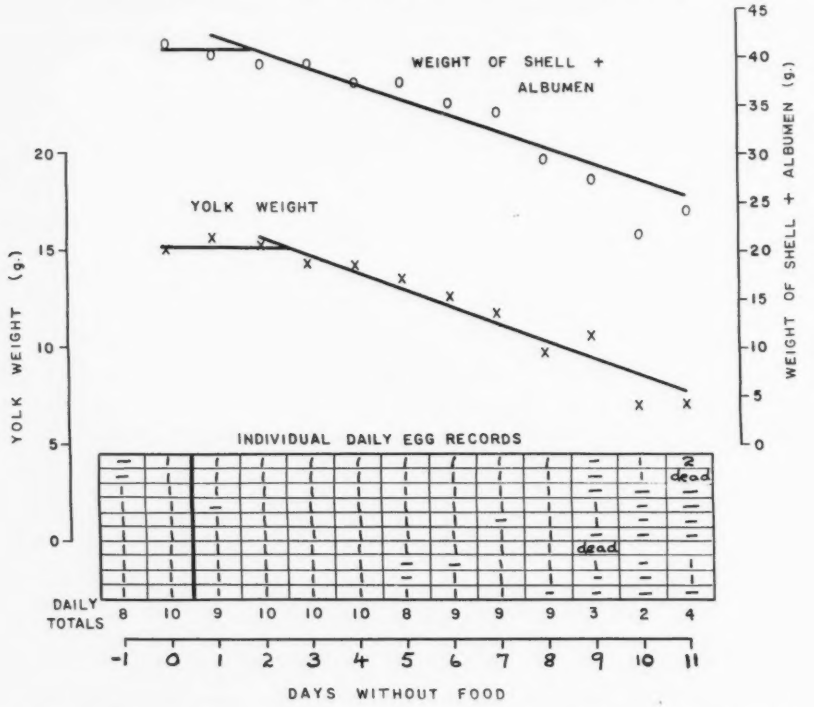
WEIGHT OF SHELL + ALBUMEN
YOLK WEIGHT
YOLK WEIGHT (g.)
WEIGHT OF SHELL + ALBUMEN (g.)
INDIVIDUAL DAILY EGG RECORDS
dead
dead
DAILY TOTALS
8 10 9 10 10 10 8 9 9 9 3 2 4
-1 0 1 2 3 4 5 6 7 8 9 10 11
DAYS WITHOUT FOOD

FIG. 3. Weight of egg components (means per egg) during starvation: data for ten highest yielding pullets treated with Chicken Anterior Pituitary powder (Experiment 4). Vertical lines indicate that an ovulation occurred on the day shown; horizontal lines show absence of ovulation. Note that one hen ovulated 2 yolks 11 days after onset of starvation.

size." After the second day, about 90% of the eggs were laid between 5 P.M. and 8 P.M., indicating that the injections made at 11 A.M. were directly responsible for the induction of ovulation. The average number of eggs laid after Day 0 by starved controls in Experiments 3 and 4 was 2.2; hence the average number induced by hormone treatment for the birds described in Table 4 was about 6 eggs.

Figure 3 shows that both yolk weight and albumen weight fell steadily when repeated ovulations were induced. The fitted linear regression lines both show significant slopes ($P < 0.001$); the regression coefficients are -0.874 gm. of yolk per day and -1.625 gm. of shell+albumen per day. All treatments showed a similar downward trend in yolk weights but in most cases the number of eggs obtained beyond Day 6 was too small to permit reliable comparisons.

The body weight losses given in Tables 3 and 4 were measured from the time of food removal to autopsy, without adjustment for gut contents or for eggs in the oviduct. An average loss of 745 gm. represents 37% of the initial liveweight of these birds. Weight losses tended to be proportional to initial liveweight (which varied from 1400 to 2500 gm.) and showed little relation, within a treatment, to the weight of eggs produced. Between treatments, it was observed in all experiments that hormone injections substantially increased the rate of weight loss and correspondingly weakened the birds so that mortality occurred first with the most "successful" treatments. In Experiment 4, six birds died before the time of autopsy: three of these were from treatment 4 (one died on Day 8 and two on Day 11); the others were from treatment 5 (Day 11), treatment 6 (Day 11) and treatment 8 (Day 10).

DISCUSSION

These experiments suggest that the primary cause of the failure of ovulation and of yolk synthesis in the starving pullet is a hormone deficiency. This situation is parallel to failure of pregnancy in the rat which occurs with various kinds of nutrient deficiency and which can be prevented by administering ovarian hormones (4, 5, 6). Whereas work on the rat was begun with vitamin deficient diets (4) and proceeded to protein-free diets (5) and later to a diet of sugar and water (6), the present study dealt with the problem of total inanition. Many fascinating experiments remain to be done with various kinds of partial nutrient deficiency. One might ask what nutrients must be provided in order to maintain life and prolong the period of yolk synthesis in the hormone-supported pullet.

Hormone treated birds in these experiments were plainly mobilizing enormous quantities of fat and substantial amounts of protein relative to their body weight. For example, the average protein output for the birds described in Table 4 was about 49 gm. in 11 days, assuming egg composition to be normal. This represents about 14% of the total protein in the

body of the pullet at the start of the experiment. In comparison, the quantity of protein in the average litter produced in 20 days by a progesterone-treated rat on a sucrose diet is about 1.3 gm., or 4% of the rat's initial body protein (6).

The use of hormones to induce a normal rate of ovulation in hens maintained on synthetic amino acid diets might help considerably towards an understanding of the function of proteins in the diet of a producing animal. For example, one might question the assumption (7, 8) that a hen receiving a lysine deficient diet stops laying abruptly because she is unable to synthesize egg proteins in these circumstances. Indeed, one might postulate that if a partial nutrient deficiency interferes directly with yolk or albumen synthesis this should be reflected in smaller or perhaps fewer eggs; but when egg production stops abruptly this should lead one to suspect interference with pituitary function.

The doses of CAP needed to maintain ovarian function in the starving bird are approximately the same as those recently found necessary in the hypophysectomized pullet (3). On the other hand, it requires 2 I.U. FSH in the hypophysectomized pullet and only 0.5 I.U. FSH in the starving pullet to maintain ovarian weight. Perhaps the reason for this is that the starving pullet secretes other pituitary hormones (TSH?) which synergize with exogenous gonadotropins.

It would seem from these experiments that avian pituitary powder is superior to mammalian gonadotropins for maintaining ovulation in the fowl. The distinction cannot be made on the basis of follicular hierarchy since overdoses of CAP were shown to produce results akin to FSH injections and no exhaustive attempt was made to arrive at optimal FSH:LH ratios or doses. However, the ability of CAP to induce repeated ovulations, with complete dependability, by intramuscular injection is in marked contrast to any known mammalian preparation. Intramuscular or intraperitoneal injections of mammalian LH preparations cause ovulation only at high doses which cannot be repeated without causing gross overstimulation of the ovary (unpublished results, this laboratory). To what extent the properties of CAP are due to the specific activity of its gonadotropin molecule(s) and to what extent they derive from the presence of other pituitary hormones is unknown.

The dose/response curve given in Figure 2 suggests that 3 mg. of this batch of CAP might have been the optimal daily dose for maintenance of normal ovarian weight. However, the possibility of testing this dose level was not pursued since the indications are that the more successfully one maintains a high ovulation rate the sooner the pullet dies. Hence the quantity of egg material produced in Experiment 4 might represent the maximum that can be obtained from a starving bird. It remains to be seen whether synthesis of yolk can continue beyond 12 days when the bird is given minimal quantities of nutrients for the support of life, or whether her

"reserves" can be exhausted so that yolk synthesis stops even though the bird remains alive and continues to receive gonadotropins.

REFERENCES

1. Hosoda, T., T. Kaneko, K. Mogi and T. Abe: *Proc. Soc. Exper. Biol. and Med.* **88:** 502. 1955.
2. Hosoda, T., T. Kaneko, K. Mogi and T. Abe: *Proc. Soc. Exper. Biol. and Med.* **92:** 360. 1956.
3. Opel, H. L.: Ph.D. Thesis, University of Illinois, 1960.
4. Nelson, M. M., W. R. Lyons and H. M. Evans: *Endocrinology* **48:** 726. 1951.
5. Nelson, M. M. and H. M. Evans: *Endocrinology* **55:** 543. 1954.
6. Kendall, K. A. and R. L. Hays: *J. Nutrition* **70:** 10. 1960.
7. Johnson, D. and H. Fisher: *J. Nutrition* **60:** 275. 1956.
8. Johnson, D. and H. Fisher: *Brit. J. Nutr.* **12:** 276. 1958.

OCCURRENCE OF THREE ELECTROPHORETIC COMPONENTS WITH MELANOCYTE-STIMULATING ACTIVITY IN EXTRACTS OF SINGLE PITUITARY GLANDS FROM UNGULATES

A. C. J. BURGERS[1]

Hormone Research Laboratory, University of California, Berkeley, California

ABSTRACT

An *in vitro* method in which the skin of a single test animal (*Anolis carolinensis*) is used, and which can be used to determine the melanocyte-stimulating activity of a large number of samples, is described. The electrophoretic behavior of the melanocyte-stimulating components present in the pituitary glands of different species of *Ungulata* was studied. It was found that single beef, pig and sheep pituitaries contain three melanocyte-stimulating components, which behaved in electrophoresis like β-glutamyl-MSH, β-seryl-MSH and α-MSH respectively. These MSH components were present in each single pituitary gland in a ratio which appeared to be more or less constant within a species and which was characteristic for that species.

THE isolation of melanocyte-stimulating hormones (MSH) from beef, pig, and sheep pituitary glands, and the determination of their structures, have been achieved during the past few years. It was discovered in the course of these various investigations that in the material prepared from the glands of any one of these animal species, different kinds of MSH are present. Two forms of MSH, namely, an α-MSH and β-seryl-MSH, were prepared from beef pituitaries (1, 2), both α-MSH and β-glutamyl-MSH were derived from pig glands (1, 2, 3, 4) and all three forms, an α-MSH, and β-seryl- and β-glutamyl-MSH's were demonstrated to exist in material of ovine origin (2, 4). In view of the fact that the starting material for these investigations consists of thousands of pooled pituitaries obtained from the slaughterhouse, there are three explanations that can be advanced for the above findings: (a) that the different types of MSH are present in a single pituitary gland, or (b) that only one type of MSH is found in any single pituitary and that the different forms come from animals of differing genotypes within the same species, or (c) that some glands containing one and other glands containing more than one type of MSH have been pooled together. To investigate these possibilities, single beef, pig, and sheep pituitary glands were studied.

With respect to bioassay, among the great variety of assay methods used

Received September 29, 1960.

[1] Permanent address: Department of Endocrinology, Zoological Laboratory, University of Utrecht, Janskerkhof 3, Utrecht, Netherlands.

to determine the melanocyte-stimulating activity of a particular substance, the *in vitro* test of Shizume *et al.* (5), which employs the isolated skin of the frog *Rana pipiens*, is the most widely used. When, however, the activity of a large number of samples must be determined, this method is unwieldy. From each frog, only four pieces of skin can be used for the test, and, because of possible variations in sensitivity to MSH among the individual test animals, each sample must be tested on four pieces of skin, each one obtained from a different animal. On the other hand, the endocrine control of pigment migration in the melanophores of an animal belonging to another class, the reptile *Anolis carolinensis*, has been demonstrated by Kleinholz (6) and an *in vitro* assay method for the detection of MSH, in which the skin of this lizard is used, has been described by Horowitz (7). Since a very distinct color change (from bright green to black brown) easily visible to the naked eye[2] is elicited in this lizard by melanocyte-stimulating substances, and since a large number of uniformly colored pieces of skin can be obtained from a single animal, *Anolis carolinensis* was chosen as the test animal for the present investigations.

MATERIALS AND METHODS

From batches of frozen pituitary glands from each of the three species studied, a single gland was picked at random. The intermediate plus posterior lobes were separated from the anterior lobe and homogenized and extracted with 0.1 M acetic acid at +4° C. The extract was centrifuged and the supernatant fluid lyophilized. A few milligrams of the lyophilized material were submitted to zone electrophoresis on starch (dimensions of trough, 1.5×0.7×40 cm.) in a pyridine-acetate buffer of pH 4.9 at 200 volts at +4° C (9). After 24 hours, the starch was cut into sections measuring 1 cm. in width and each section was placed in a test tube and eluted with 3 ml. of distilled water. A portion of each eluate was transferred to a fresh test tube and dried over P_2O_5.

Biological assay for melanocyte-stimulating activity was carried out with the skin of the lizard *Anolis carolinensis*. The animals, weighing 3–5 gm. each, were kept in a terrarium at 24° C and were fed on *Tenebrio molitor* larvae. Before each experiment, a number of the animals were placed in a green jar and exposed to continuous illumination for at least 24 hours. From these animals, the one whose color was the brightest green was chosen; this animal was decapitated, the extremities and tail were cut off, a medioventral incision was made, and the skin was peeled off. The isolated skin was transferred to a Petri dish filled with Ringer's solution.[3] After it had been in the solution for one hour, the bright green skin from the dorsal lateral sides of the body was cut into numerous small pieces (about 70). The very thin skin which covers the joints of the

[2] The photoelectric methods used by Horowitz (7) and by Novales (8) for quantitative measurement of the color change in *Anolis* skin are impractical here, since in this instance such a large number of samples must be tested on the skin of the same lizard, and since these two methods require large pieces of skin.

[3] It is interesting that when a piece of skin is added to a Ringer's solution which contains MSH, its bright green color changes to brown, and also that it regains its initial color when replaced in Ringer's solution without hormone. Skin pieces which had been stored floating on Ringer's solution in a closed Petri dish at 20° C, for as long as 72 hours, proved to be still responsive to MSH (see also 7). In our actual experimental series, however, each experiment was finished within 5 hours after decapitation.

extremities and the skin from the neck region, which has somewhat larger scales than does the skin of the rest of the body, were not used in the assay.

To each of the 40 test tubes containing the dried material obtained from the starch electrophoresis, 5 ml. of Ringer's solution was added. The contents of each test tube were poured into a separate 10 ml. beaker, and the solution was poured back and forth 3 times to wash out all the material from the test tube. Then, to the 5 ml. of fluid in each beaker, a piece of lizard skin was added. The color change of the piece of skin in each beaker was followed with the naked eye. The beakers were placed on a black background and illuminated by a 100 w. bulb suspended 1 meter above them. When a color change from bright green to brown was noted, 5 ml. of Ringer's solution and a fresh piece of bright green skin was added to the solution. When this second piece also turned brown, a quantity varying from 5 to 0.01 ml., depending on the rapidity and intensity of the color change, was transferred by pipetting from the 10 ml. solution into a second beaker and was diluted with Ringer's solution to make a volume of 10 ml. A fresh piece of lizard skin was then added to this new solution. This procedure was repeated until a concentration was obtained in which a weak color change from bright green to a definite brownish green was elicited in a piece of *Anolis* skin within half an hour, and below which a color change could no longer be observed in a fresh piece of skin if an additional dilution with 5 ml. more of Ringer's solution was made. In all these experiments, the color of the piece of skin in each of the hormonal solutions was compared with that of a piece of skin in Ringer's solution alone. The experiments were carried out under standardized conditions of light as described above and at room temperature (24° C).

Highly purified α-MSH and β-seryl-MSH from bovine pituitaries (10, 11) and β-glutamyl-MSH from porcine glands (12) and α-ACTH from bovine pituitaries (13) were prepared in this laboratory by methods previously described and were used as controls.

RESULTS

When the melanophore-stimulating activity of each of the 40 samples obtained from starch electrophoresis of single bovine pituitary glands was tested on *Anolis* skin, three distinctly different zones of activity were found to be present. The zones of highest MSH activity were located 8, 14, and 21 cm. from the origin. For convenience, we shall designate the three components as A, B, and C, respectively. The same result was obtained in all of the 5 experiments carried out with single bovine pituitary glands. The data from a typical experiment are given in Figure 1.

In the next series of experiments, extracts of single intermediate plus posterior lobes of sheep and pig were submitted to electrophoresis in separate troughs under the same conditions as the material of bovine origin. In the samples from single sheep glands and single pig glands, three zones of MSH activity were again found, located at the same distances from the origin (8, 14, and 21 cm.) as the components from bovine pituitaries. The experiments were repeated 4 times more with single sheep and pig glands, and in each instance a similar result was obtained (Figs. 2 and 3).

To compare the mobilities of the active components from the single pituitary glands with those of the various froms of MSH of known chemical structure, highly purified α-MSH (bovine origin), β-seryl-MSH (bovine origin) and β-glutamyl-MSH (porcine origin) were submitted to electrophoresis in separate troughs; the parallel experiments were carried out simultaneously with and under identical conditions as the lyophilized ex-

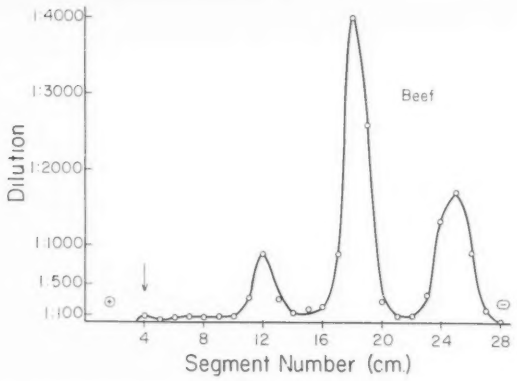

FIG. 1. Zone electrophoresis on starch of an extract of a single beef pituitary (intermediate-posterior lobes only). The experiment was carried out in a pyridine-acetate buffer of pH 4.9 at 200 volts for 24 hours at +4° C. The eluate from each segment was assayed for melanotropic activity by the *Anolis* skin method. The material was applied in the position as indicated by the arrow.

tract from the single glands. It was found that α-MSH migrated a distance of 21 cm., β-seryl-MSH, 14 cm., and β-glutamyl-MSH, 8 cm. from the origin, the same distances respectively as the three components that were observed under identical conditions in single ox, sheep and pig pituitaries.

Finally, to investigate whether the mobilities of the known MSH's change when they are submitted to electrophoresis together instead of in separate troughs, a mixture of the 3 highly purified MSH preparations was submitted to electrophoresis in a fourth trough, along with the other three troughs containing the single components. No significant differences in mobilities were noted between the components which were run separately and the mixture.

It is known that ACTH possesses an intrinsic melanocyte-stimulating activity (14); hence, it was thought that it would be of interest to compare the electrophoretic mobilities of the ACTH components that possess melanocyte-stimulating activity with those of the MSH components that

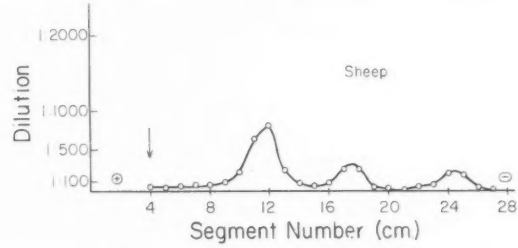

FIG. 2. Zone electrophoresis on starch of an extract of a single sheep pituitary (intermediate-posterior lobes only). Conditions were exactly the same as those shown in Figure 1.

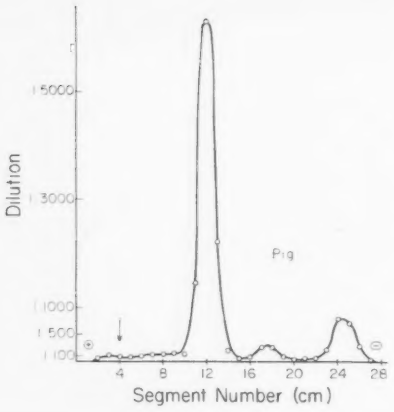

FIG. 3. Zone electrophoresis on starch of an extract of a single pig pituitary (intermediate-posterior lobes only). Conditions were exactly the same as those shown in Figure 1.

were obtained in this electrophoretic system. None of the peaks obtained in the previous experiments appeared within the area in which the melanocyte-stimulating activity of the ACTH was located.

These results demonstrate that different components with melanocyte-stimulating activity are present in a single pituitary gland, and it may also be concluded that not only sheep pituitaries but also single beef and single pig pituitaries contain 3 forms of MSH. It is likely that the component which migrated 8 cm. from the origin in the experiments with single pig glands is β-glutamyl-MSH and the material which migrated 14 cm. from the origin in the experiments with single beef glands is β-seryl-MSH.

Although the pituitaries of all three ungulate species tested were thus all found to contain the three different components with MSH activity, within each species the degree of dilution necessary to induce the threshold reaction in a piece of lizard skin is different for each of the three active components, and moreover, the ratios of the activities of each of the three peaks (Figs. 1–3) as reflected in these dilutions appear to be more or less characteristic for each species. On the basis of these ratios, it can be concluded that Component B, which has the same mobility as β-seryl-MSH, is the main component in the extracts from beef glands, whereas the main component in the porcine material is Component A, which has the same mobility as β-glutamyl-MSH. In the case of the sheep glands, the 3 peaks are more nearly equal, with no distinct major component. Since components A, B, and C each have different specific activities, the differences in these ratios of activities within each species do not necessarily mean the various components are present in differing amounts in the extracts.

DISCUSSION

On the basis of the results of the experiments reported herein, it can be concluded that the first of the explanations offered in the Introduction,

to the effect that different types of MSH are present in a single pituitary gland, is the correct one in all instances. Moreover, the results obtained in the present study with single sheep pituitaries are in agreement with previous observations (2, 4) that extracts of pooled sheep pituitaries contain both β-seryl-MSH and β-glutamyl-MSH in addition to the α-MSH. The apparent discrepancy between beef and pig glands on the one hand, from which only two types of MSH have been isolated (1, 2), and sheep glands on the other, in which three types were found, was in fact not real, since three different active components were found to be present in the extracts of all the single glands from each of the animal species investigated. As can be seen in Figures 1 and 2, the least active component in extracts of single beef glands is Component A, the component which behaves like the glutamyl form of the hormone, and the least active in extracts of single pig glands is Component B, the component which behaves like the seryl form. This may explain the fact that these substances were not detected in previous investigations.

In the findings of Lee and Lerner (3), the main component of pig pituitaries was α-MSH; furthermore, Geschwind and Li (2, 4) reported the ratio with respect to content of β-glutamyl-MSH to β-seryl-MSH in sheep pituitaries as 1:4. The results of the present investigations are not in agreement with these reports (2, 3, 4). These discrepancies in quantitative data may be explained by differences in extraction methods and bioassay techniques.

Acknowledgments

This work was supported in part by a grant (RG 2907) from the National Institutes of Health of the United States Public Health Service. The author wishes to thank Professor C. H. Li for placing at his disposal the highly purified hormone preparations, for suggesting this problem and for his continued interest and advice. The author also wishes to express gratitude to the Rockefeller Foundation for a fellowship, under the tenure of which this work was carried out (1959–1960).

REFERENCES

1. Li, C. H.: *Laboratory Investigations* **8:** 574. 1959.
2. Geschwind, I. I.: *Comparative Endocrinology*, Proc. Columbia U. Sympos. on Comp. Endocrin., A. Gorbman, ed. John Wiley and Sons, Inc. 1959. p. 421.
3. Lee, T. H. and A. B. Lerner: *J. Biol. Chem.* **221:** 943. 1956.
4. Li, C. H.: Symposium on Protein Structure (Neuberger, A., ed.) Methuen and Co., Ltd. London, Eng. (1958). p. 302.
5. Shizume, K., A. B. Lerner and T. B. Fitzpatrick: *Endocrinology* **54:** 553. 1954.
6. Kleinholtz, H.: *J. Exper. Biol.* **15:** 492. 1938.
7. Horowitz, S. B.: *J. Cell. and Comp. Physiol.* **51:** 341. 1958.
8. Novales, R. R.: *Physiol. Zool.* **32:** 15. 1959.
9. Fønss-Bech, P. and C. H. Li: *J. Biol. Chem.* **207:** 175. 1954.
10. Dixon, J. S. and C. H. Li: *Fed. Proc.* **18:** 848. 1959.
11. Geschwind, I. I., C. H. Li and L. Barnafi: *J. Am. Chem. Soc.* **79:** 1003. 1957.
12. Geschwind, I. I., C. H. Li and L. Barnafi: *J. Am. Chem. Soc.* **78:** 4494. 1956.
13. Li, C. H. and J. S. Dixon: *Science* **124:** 934. 1956.
14. Li, C. H.: *Adv. Prot. Chem.* **11:** 101. 1956.

PRODUCTION OF ANTIBODIES TO HUMAN PITUITARY GROWTH HORMONE IN THE RAT

N. R. MOUDGAL AND CHOH HAO LI

The Hormone Research Laboratory, University of California, Berkeley, California

ABSTRACT

The rat has been shown to be capable of forming antibodies to human growth hormone. Hypophysectomy apparently has no determining influence on the type of antibody formed.

A RECENT report (1) from this laboratory has shown that when growth hormone obtained from either human or monkey pituitaries was injected into hypophysectomized rats, body-weight gains were seen only during the first ten days and that after this period no growth-promoting effect was observed even if the daily dosages were increased. Contrariwise, either bovine or cetacean growth hormone exerted a growth-promoting effect for as long as 90 days. Since it has been demonstrated that human growth hormone (2) is more potent as an antigen in the rabbit than is the bovine hormone (3), it was suspected that antibodies were produced in the rat by the injections of the human growth hormone, counteracting the biological effect of the hormone itself. In this communication, data are presented which substantiate this hypothesis.

EXPERIMENTAL

The human pituitary growth hormone was isolated by the procedure previously described (4, 5). Adult female rats of the Long-Evans strain, either normal (140 days old) or hypophysectomized at 28 or 140 days of age were used as experimental animals. In the initial series of experiments, a group of 30 rats hypophysectomized at 28 days of age were injected, beginning on the 14th day after operation and continuing for a period of 49 days, with a daily dose of 0.025 mg. of human growth hormone by the intraperitoneal route. After being permitted to rest for one week, the animals were then given a booster injection in the amount of 0.10 mg., and were bled one week later. The hormone was administered in saline solution, and an adjuvant was not used.

In the second series of experiments, 3 normal adult female rats were injected intraperitoneally with a daily dose of 0.2 mg. of human growth hormone for 20 days, and, after being permitted to rest for one week, they were then given a booster dose of 1 mg. of the hormone and were bled 7 days after this final injection. The hormone administered to this group was also in saline solution without adjuvant.

For the third series of experiments, 6 normal adult female rats were divided into two groups of 3 each. The animals in one of these groups were hypophysectomized and immunization was begun 14 days after the operation. The animals in the other group were left intact, and were started on the immunization schedule at the same time as the

Received October 3, 1960.

hypophysectomized animals. During the first week, 2 injections of 200 μg each of human growth hormone in saline solution were administered 3 days apart, with half of each injection given by the subcutaneous route and half by the intraperitoneal route. During the second week, one injection of 400 μg of human growth hormone in the adjuvant Bayol-Arlacel was given, followed in the third week by another injection of 200 μg of hormone in the same adjuvant. The animals were permitted to rest during the 4th and 5th weeks, and in the 6th week they were given a booster injection of 1 mg. of human growth hormone in saline solution. The animals were bled the following week. The sera from the animals within each of these groups were pooled. Since the sera showed a weak reaction with normal human serum in a dilution of 1:10, the antibodies to the serum proteins were removed by the addition of small amounts of diluted normal human serum, and these absorbed sera were used in all subsequent experiments. The sera of the normal and the uninjected hypophysectomized rats served as controls.

Qualitative precipitin ring tests were performed by layering 0.2 ml. of antiserum under 0.2 ml. of a saline solution containing 0.01 mg. of human growth hormone and incubating for one hour at 37°. The procedure followed for the quantitative precipitin tests was essentially that of Heidelberger and Kendall as described earlier (6). Double diffusion tests in agar-gel according to the method of Ouchterlony were also performed as previously described (6). The antisera were assayed for anti-hormone activity in hypophysectomized rats by the tibia test (7).

RESULTS

The sera obtained from the animals in the first series of experiments, those that were hypophysectomized at 28 days of age and given long-term treatment with human growth hormone, contained no antibodies of the precipitating type, as evidenced by the absence of any precipitin bands in the qualitative precipitin ring test and after diffusion in agar-gel (Fig. 1). The sera from this group, however, was capable of significantly neutralizing the growth hormone activity measured by the tibia test in hypophysectomized rats (Table 1), indicating the presence of soluble antibodies. On the other hand, the sera from the normal adult female rats in the second experimental group did contain antibodies of the precipitating type (Fig. 1) and were also able effectively to neutralize growth hormone activity in hypophysectomized rats (Table 1).[1]

It was deemed of interest at this point to ascertain whether removal of the pituitary influences the type of antibody to human growth hormone formed. With this in view, a third series of experiments with sera from adult rats, both hypophysectomized and intact, was undertaken. Results of diffusion in agar-gel (Fig. 2) and of qualitative precipitin ring tests

[1] In a recent experiment where rats hypophysectomized at 28 days of age were immunized with a total dose of 2 mg. of human growth hormone given in Freund's adjuvant (complete), it was observed that the sera of these animals contained the precipitating type of antibodies. This would seem to indicate that the differences observed earlier in the type of antibody produced in the 28-day and in the 140-day hypophysectomized rats are not due to any age factor, but rather to variation in the immunological technique and to the dosage of human growth hormone (added in proof, Jan. 17, 1961).

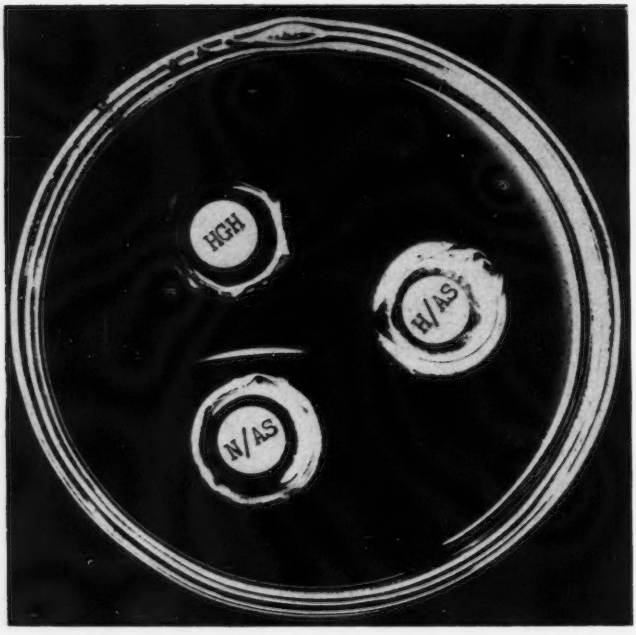

FIG. 1. An Ouchterlony agar diffusion pattern showing interaction between human growth hormone (HGH) and normal rat serum to HGH (N/AS), and between HGH and rat antiserum to HGH from young hypophysectomized rats (H/AS).

showed the presence of the precipitating type of antibodies in the serum from both groups. Quantitative precipitin curves for the animals of both groups were very similar, and the equivalence point in each case was reached when 50 μg of the antigen, human growth hormone, was added. A typical precipitin curve for the human growth hormone-rat antiserum system is shown in Figure 3.

TABLE 1. THE INFLUENCE OF RAT ANTISERUM TO HUMAN GROWTH HORMONE (HGH) ON THE BIOLOGICAL ACTIVITY OF HGH AS DETERMINED BY THE STANDARD 4-DAY TIBIA TEST

Group*	No. of rats	Tibia plate width
		$\mu \pm$S.E.
Saline	10	157 ± 2
HGH	12	244 ± 6
HGH+N/AS	6	205 ± 4
HGH+H/AS	6	$206 + 3$
HGH+rat serum (H)	3	237 ± 5

* Human growth hormone (HGH) was given in a dosage of 0.010 μg daily for 4 days; hypophysectomized rat antiserum (H/AS), 0.5 cc. daily; normal rat antiserum (N/AS), 0.25 cc. daily; serum from hypophysectomized rats (rat serum (H)), 0.5 cc. daily.

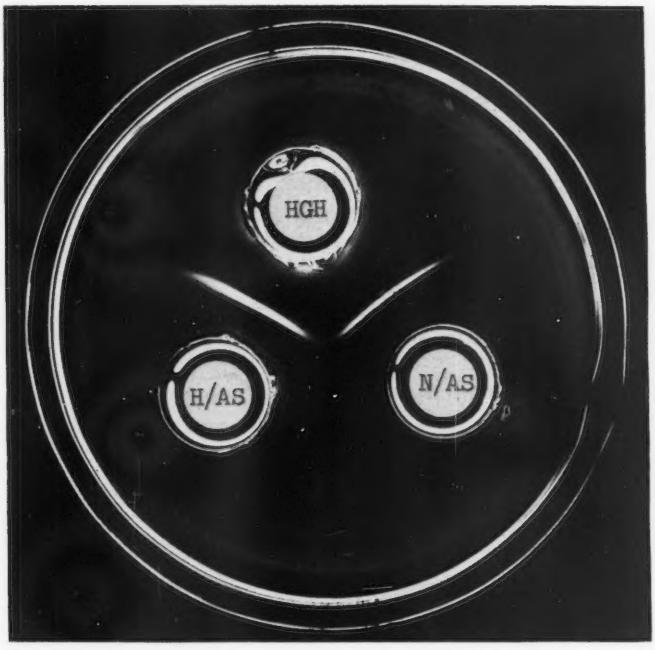

FIG. 2. An Ouchterlony agar diffusion pattern showing interaction between human growth hormone (HGH) and normal rat serum to HGH (N/AS), and between HGH and antiserum to HGH from adult hypophysectomized rats (H/AS).

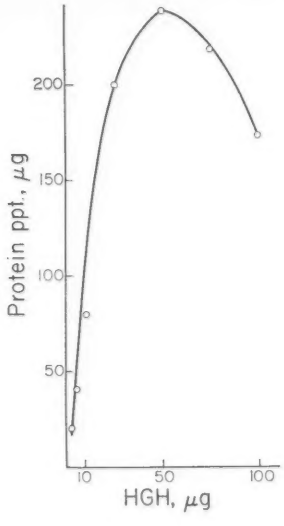

FIG. 3. Quantitative precipitin curve obtained from the reaction of rat antiserum (0.5 ml.) to human growth hormone (HGH) with HGH.

DISCUSSION

It has been unequivocally demonstrated by the results of these studies that the rat is able to form antibodies to human growth hormone. The cessation of body weight gain after 10 days observed in the long-term experiments with the primate hormones (1) mentioned above, may apparently be accounted for by neutralization of the growth hormone effect by these antibodies. The presence of antibodies of the precipitating type in serum from both normal and hypophysectomized adult rats and the similarity between the precipitin curves and equivalence points observed for the antigen-antibody systems of both, leads to the conclusion that hypohysectomy of the animal does not have any determining influence on the type of antibody formed in the animal. This conclusion was supported by results of unpublished studies with human gamma globulin as antigen instead of growth hormone, wherein adult hypophysectomized and intact rats were both observed to form antibodies of the precipitating type. It may be of interest to note here that the equivalence point for the human growth hormone-rabbit antibody system is also located at 50 μg., and furthermore, that it was observed in the present experiments that the rabbit

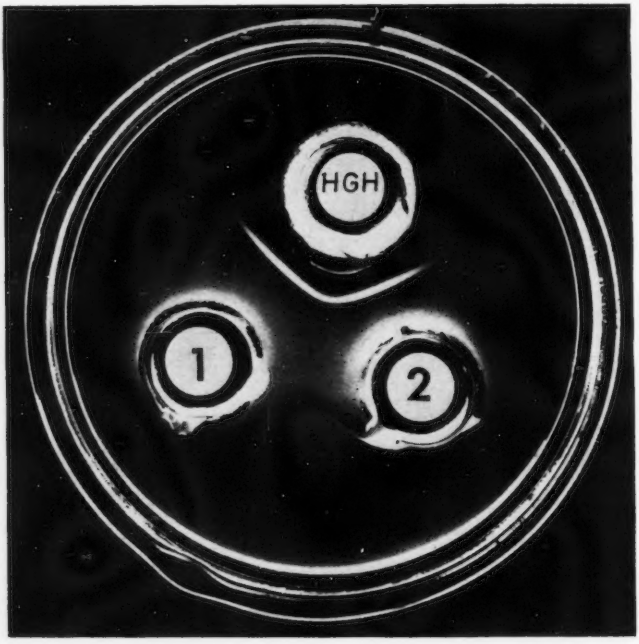

FIG. 4. An Ouchterlony agar diffusion pattern showing cross reaction between rabbit and rat antibody to human growth hormone (HGH). 1: rat antibody, 2: rabbit antibody.

and the rat antibodies to human growth hormone cross-react with one another (Fig. 4). It has also been observed that human and monkey growth hormones cross-react with one another when rat antiserum to the human hormone is used; these results are in agreement with earlier data obtained with the rabbit antiserum (6).

Acknowledgments

The authors wish to thank Charles W. Jordan, Jr., for his able assistance during the course of these investigations. This work was supported in part by grants from the American Cancer Society, New York, and the Albert and Mary Lasker Foundation.

REFERENCES

1. Li, C. H., H. Papkoff, C. W. Jordan, Jr.: *Proc. Soc. Exper. Biol. and Med.* **100:** 44. 1959.
2. Hayashida, T., C. H. Li: *Science* **128:** 1276. 1958.
3. Hayashida, T., C. H. Li: *Endocrinology* **65:** 994. 1959.
4. Li, C. H., H. Papkoff: *Science* **124:** 1293. 1956.
5. Li, C. H.: *Fed. Proceedings* **16:** 776. 1957.
6. Li, C. H., N. R. Moudgal, H. Papkoff: *J. Biol. Chem.* **235:** 1038. 1960.
7. Greenspan, F. S., C. H. Li, M. E. Simpson, H. M. Evans: *Endocrinology* **45:** 455. 1949.

THE HISTOCHEMICAL DISTRIBUTION AND METABOLIC SIGNIFICANCE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY, GLYCOGEN AND LIPID IN THE STIMULATED ADRENAL CORTEX[1]

RICHARD B. COHEN

Department of Pathology, Harvard Medical School, and the James Homer Wright Pathology Laboratories of the Massachusetts General Hospital, Boston, Massachusetts

ABSTRACT

The adrenal cortices of normal and hypophysectomized rats subjected to cold stress of varying duration were studied using histochemical methods to determine the distribution of glucose-6-phosphate dehydrogenase activity, glycogen, and lipids. It was found that glucose-6-phosphate dehydrogenase activity did not vary in distribution or intensity in response to stress in either the normal or hypophysectomized animals, but remained concentrated in the reticular and fascicular zones. Glycogen in the normal animal, although distributed in the same areas as the enzyme, was depleted rapidly by stress, often disappearing from the cortex after only 30 minutes of stress. This was much more rapid than was the response of lipid to stress. Glycogen was usually well on its way to repletion before cortical lipid was at its lowest ebb. Neither glycogen nor fat responded to stress in the hypophysectomized animals. It is suggested that glycogenolysis initiated by ACTH provides substrate for the glucose-6-phosphate dehydrogenase, this setting into motion the initial steps in the pentose phosphate pathway of carbohydrate metabolism. This provides the energy and reduced triphosphopyridine nucleotide required for steroid synthesis and results in the utilization of cortical lipid stores. The histochemical method provides distributional data pertinent in the evaluation of a theory of ACTH function derived from the use of other technics.

IN THIS paper we report an investigation using histochemical methods to determine the distribution of glucose-6-phosphate dehydrogenase activity, glycogen, and lipid in the adrenal cortex of normal and hypophysectomized rats which have been submitted to a standard stress. The results are correlated and interpreted in the light of some recently advanced theories of ACTH function in cortical metabolism.

MATERIALS AND METHODS

Sixty normal and 30 hypophysectomized male and female white rats were used. The animals were kept on a standard laboratory diet and acclimatized to the animal room for at least 1 week. The hypophysectomized animals were included in the experiment

Received October 12, 1960.

[1] This research was supported by Grant C-3424 of the National Cancer Institute, National Institutes of Health.

when they were from 21 to 28 days postoperative. In each experiment a series of rats, under identical conditions, were submitted to a temperature of 4° C for periods of time varying from 15 minutes to 24 hours in a cold room. Each series of normal and hypophysectomized rats exposed to cold included an unexposed control. At the completion of the prescribed period of stress the animals were sacrificed by a blow on the head. Both adrenals were immediately removed, and frozen sections 20 micra thick were cut in a cryostat (−18° C). Each section used constituted a cross section of the gland.

Glucose-6-phosphate dehydrogenase activity was demonstrated in unfixed sections by a technic previously described using nitro-B.T.[2] as hydrogen acceptor and indicator of the site of enzyme activity (1, 2).

Glycogen distribution was demonstrated in sections previously fixed for 20 minutes in cold (4° C) absolute alcohol. These were then submitted to the periodic acid Schiff (PAS) reaction. To prove the identity of PAS positive material as glycogen similarly prepared sections were submitted to diastase digestion prior to application of the PAS reaction. Alcohol fixation removed any confusion that might have been offered by cortical lipids.

In addition parallel sections were fixed for from one to three hours in 10% formalin and reacted with the Sudan IV stain for lipids.

RESULTS

Neither stress nor hypophysectomy affected the distribution of glucose-6-phosphate dehydrogenase activity in the adrenal cortex. In the cortices of both stressed and control normal rats enzyme activity was concentrated in the cytoplasm of cells in the fascicular and reticular zones. The zona glomerulosa was sharply demarcated in these sections by its comparative lack of activity (Fig. 1). In the hypophysectomized animals the inner zones were markedly narrowed measuring no more than one-third to one-half their width in the normal animal. The fasciculata and reticularis that remained, however, showed considerable enzyme activity, and by the rough

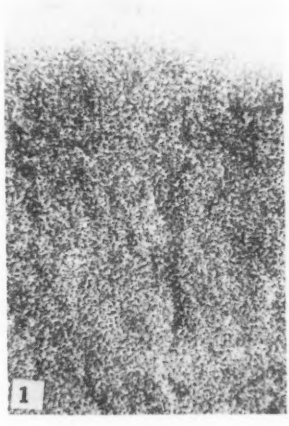

FIG. 1. Glucose-6-phosphate dehydrogenase activity in the normal cortex is concentrated in the fascicular and reticular zones with comparatively slight activity in the glomerulosa. ×100.

[2] 2,2′ Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) Ditetrazolium; Dajac Laboratories, Philadelphia.

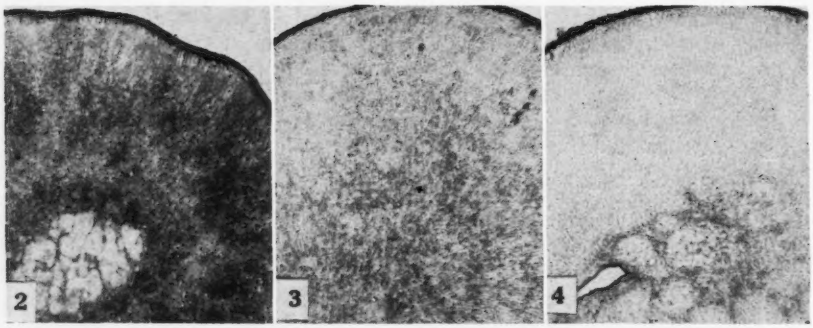

FIG. 2. Glycogen distributed in the fascicular and reticular zones of the normal control. The glomerulosa contains little glycogen. Dense staining of the capsule and of the reticular network of the medulla is diastase resistant; therefore not glycogen. PAS ×60.

FIG. 3. Glycogen distribution in the normal animal after 15 minutes exposure to cold showing regression of glycogen particularly from outer fascicularis. Staining of capsule is diastase resistant. PAS ×80.

FIG. 4. Complete depletion of glycogen from the fascicular and reticular zones after 40 minutes of cold stress. The glomerulosa still retains its original small concentration of glycogen. Capsular and medullary staining are diastase resistant. PAS ×60.

methods of quantitation available to us, appeared proportionate to that of the normal gland.

In the normal control animal glycogen was richly distributed in the cytoplasm of the cells of the fascicular and reticular zones. The glomerulosa contained considerably less of this substance. The distribution of glycogen paralleled that of glucose-6-phosphate dehydrogenase activity (Fig. 2). After 15 minutes of stress considerable depletion of glycogen was evident in the outer and mid-fascicular areas (Fig. 3), and in the animals stressed from 30 minutes to 1 hour glycogen was no longer discernible in the cortex (Fig. 4). The small concentration of glycogen in the glomerular zone was not appreciably affected by this stimulus. As the duration of cold exposure increased glycogen reaccumulated and in 4 to 5 hours was clearly evident in the reticular zone and by 6 to 7 hours was reestablished in roughly the same distribution and quantity as in the control. In the hypophysectomized animal glycogen was prominent in the narrow inner zone remnant and appeared to undergo little or no variation in response to stress.

The response of the cortical lipid of the normal and hypophysectomized animals to stress conformed to that observed by others (3). In the normal animals gradual depletion of lipid was first evident in the reticular zone with extension of this loss into the fascicular zone. Lipid loss appeared to be maximal after 3–5 hours of exposure (Fig. 5). The glomerulosa remained rich in lipid which did not fluctuate. The narrowed fascicular and reticular zones of the hypophysectomized animals contained somewhat reduced

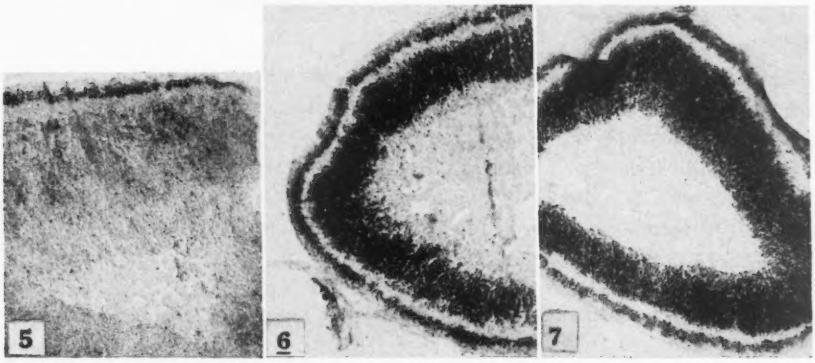

FIG. 5. Lipid depletion of the reticular and fascicular zones in the normal animal exposed to 5 hours of cold. Lipid of the glomerular zone remains intact. Sudan IV ×60.

FIG. 6. Lipid distribution in the cortex of the control hypophysectomized animal. Sudan IV ×60.

FIG. 7. Lipid distribution in the hypophysectomized animal exposed to 5 hours of cold. There is little difference in lipid content and distribution between this adrenal and the control in Fig. 6. Sudan IV ×60.

lipid stores but considerable lipid was still present and this did not fluctuate appreciably in response to stress (Figs. 6, 7).

DISCUSSION

The adrenal cortex is one of the mammalian tissues which utilizes the alternative mechanism of the pentose phosphate pathway for the metabolism of carbohydrate to a greater extent than do most other tissues (4, 5). This may be correlated with the evidence provided by Haynes (6, 7) who showed that adrenocorticotrophin (ACTH) may act, at least in part, to promote steroid synthesis through a stimulation of adrenal phosphorylase activity. This results in glycogenolysis and the production of glucose-1-phosphate and glucose-6-phosphate. The latter substance is oxidized via a triphosphopyridine (TPN) mediated dehydrogenation to 6-phosphogluconic acid with the reduction of TPN to TPNH. The acid is in turn oxidatively decarboxylated using TPN as coenzyme with the production of ribulose-5-phosphate and more TPNH. From the point of view of adrenal cortical metabolism, this is significant in that it sets up the basis for a hypothesis into which ACTH activity may be logically integrated as the stimulant which sets into motion a metabolic mechanism producing energy and which is at the same time a TPNH generator. TPNH is an important reductant in the enzymatic hydroxylations of the steroid molecule, and more recently has been implicated at a more proximal point in steroid synthesis, having been found necessary for the splitting of the cholesterol side chain (8).

In a previous paper we have utilized a histochemical technic to localize glucose-6-phosphate dehydrogenase activity in the adrenal cortex of the resting rat. The enzyme was very active in the fascicular and reticular zones, and comparatively low in the glomerulosa (2). The finding of high activity of glucose-6-phosphate dehydrogenase in the inner zones is logical since it is this area which responds most actively to acute stress and ACTH administration. It is here that we would expect to find a requirement for a mechanism which would produce rapidly available energy and at the same time generate TPNH.

In the animals observed in this study neither stress nor hypophysectomy appears to affect the distribution or intensity of activity of glucose-6-phosphate dehydrogenase. Glycogen, although concentrated in the same zones as the enzyme, is rapidly affected by stress and markedly depleted within 15 minutes of the onset of exposure to cold (9). Complete disappearance of glycogen from the fascicular and reticular cortex occurs in 30 minutes to an hour. The cortices of the hypophysectomized animals although well supplied with glycogen show little or no response of this glycogen to stress. Thus, ACTH does not appear to affect appreciably the intensity or distribution of glucose-6-phosphate dehydrogenase activity, but perhaps functions through its stimulation of glycogenolysis to provide a ready supply of substrate for the enzyme. In the hypophysectomized animal which lacks ACTH the stimulus to glycogenolysis is absent and though apparently ample enzyme, lipid and glycogen are present, the mechanism is not initiated (Fig. 8). The relationship between the fluctuation of lipid and

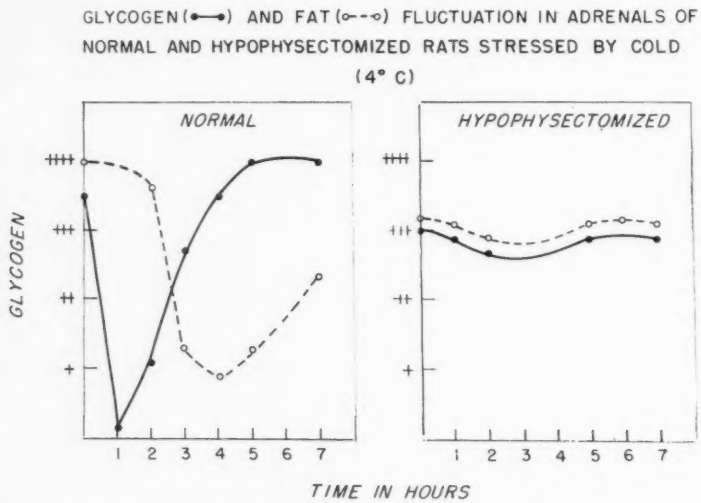

FIG. 8. These are representative curves of glycogen and lipid fluctuation in response to cold in the cortices of normal and hypophysectomized animals.

glycogen in response to stress in the normal animal is interesting and is outlined in the graph in Figure 8. The glomerulosa contains the least glycogen in the cortex and this remains stable despite stress. The next lowest concentration of glycogen appears to be in the outer fascicular zone and this is rapidly depleted in response to stress. Greatest concentrations are present in the mid-fascicular and reticular areas and glycogen persists for the longest time in this area of the stressed cortex. Lipids, on the other hand, are least concentrated and disappear most rapidly from the reticular and inner fascicular zones. The fats of the cortex do not become markedly depleted until glycogen is well on its way to repletion. If a portion of this lipid represents steroid precursor it may be postulated that the initial breakdown of glycogen provides energy and TPNH for rapid synthesis of steroids which are secreted in the early stages of stress but do not reduce lipid stores sufficiently to be discernible on histochemical examination. As the stress is prolonged into hours glycogen may be reformed perhaps at an accelerated rate keeping pace with the increased glycogenolysis. This would provide energy necessary for maintenance of the increased steroid synthesis that ultimately consumes lipid stores. By 6 or 7 hours of exposure to cold demonstrable glycogen reaccumulates in the cortex, and it may be assumed that despite the continued stress the rate of synthesis exceeds the rate of breakdown and consumption. In 24 hours or less the lipid stores also return to apparently normal levels. Both elements at this stage may, however, be turning over at an accelerated rate which cannot be measured by the histochemical technic.

In this investigation by the use of histochemical methods which give localizing and distributional data we have attempted to provide supplementary information for consideration in evaluating a concept of ACTH function in adrenal cortical metabolism. None of the data accumulated in these experiments preclude the possibility that ACTH may act at more than one level in cortical metabolism.

REFERENCES

1. Nachlas, M. M., D. G. Walker and A. M. Seligman: *J. Biophys. & Biochem. Cytol.* **4**: 467. 1958.
2. Cohen, R. B.: *Proc. Soc. Exper. Biol. & Med.* **101**: 405. 1959.
3. Greep, R. O. and H. W. Deane: *Ann. N. Y. Acad. Sci.* **50**: 596. 1949.
4. Fruton, J. S. and S. Simonds: *Gen. Biochem.* John Wiley and Sons, New York. 1958.
5. Kelly, T. L., E. D. Nielson, B. R. Johnson and C. S. Vestling: *J. Biol. Chem.* **212**: 545. 1955.
6. Haynes, R. C., Jr. and L. Berthet: *J. Biol. Chem.* **225**: 115. 1957.
7. Haynes, R. C.: *J. Biol. Chem.* **233.2**: 1220. 1958.
8. Halberstron, J. D. K., J. Eichhorn and O. Hechta: *Arch. Biochem. & Biophys.* **85**: 287. 1959.
9. Planel, H. and A. Guilhem: *C. rend. Soc. biol.* **152**: 165. 1958.

NOTES AND COMMENTS

ATROPHY OF THE FETAL MOUSE ADRENAL FOLLOWING DECAPITATION IN UTERO

ABSTRACT

In decapitated mouse fetuses and in litter-mate controls, the effects of the decapitation upon the adrenal cortex were determined by volumetric and histologic studies. The results were essentially like those heretofore reported for the rat. The lipid-free zone of the rat cortex was not observed, however, in mouse fetuses, neither in decapitated fetuses nor in controls. In general, the adrenals of decapitated fetuses were smaller than those of controls, both in absolute volume and in the ratio "adrenal volume/body weight." Microscopically, the adrenals of decapitated fetuses differed from those of controls in the following respects: the cortex was narrower; the cortical cells were smaller, less eosinophilic and more vacuolated; the lipid droplets in cells were larger and more widely separated from each other. These volumetric and histologic changes varied more or less directly with the duration of the decapitation. They were very conspicuous when the experimental period included the interval between days 18 and 19 of pregnancy.

In order to study the role of the hypophysis in the adrenal development in fetal rat several workers removed the fetal hypophysis by decapitating the fetus, and found that decapitation resulted in a retardation of adrenal growth (1–6) and that this retardation could be prevented by injected ACTH (2, 6). As the period of absence of hypophysis lengthened, the changes in the weight and histology of the adrenal were accentuated (4). It would be of special interest to know whether or not the retardation of growth of the fetal adrenal after decapitation takes place in species other than the rat and rabbit (7).

The present study was undertaken in order to determine whether the adrenal of the fetal mouse would undergo atrophy after decapitation in a fashion similar to that described in the rat. The experimental plan was like that of Cohen (4) in that the decapitation was performed on different days of pregnancy and also in that the duration of the experiments after the decapitation was varied.

MATERIALS AND METHODS

Female albino mice of the bc strain (8) were placed with males overnight, and were examined the next morning for the presence of copulation plugs. The day of observation of plug was counted as day 1 of pregnancy.

Pregnant females were subjected to laparotomy for the decapitation of fetuses *in utero*, by a method which has been described (9). One or 2 fetuses per litter were decapitated, and this operation was performed on 3 different days of pregnancy, i.e., on days 16, 17 and 18. The autopsies were performed 1 or 2 or 3 days after the operation. Intact (non-decapitated) litter-mates served as controls, and they were decapitated just before weighing (Table 1). All fetuses were weighed individually.

The left adrenal together with the kidney was fixed in Bouin's fluid, embedded in paraffin and sectioned in series at 10 μ. The sections were stained with hematoxylin and eosin. From the stained sections, the volume of the adrenal was determined by the paper-weight method. Every 4th section was traced onto ledger paper with the aid of a camera lucida.

The right adrenal was fixed in 10% formalin, frozen and sectioned at 20 μ. The sections were stained with Sudan black B for sudanophilic lipids.

RESULTS

The results were essentially like those previously reported in the rat, and the changes in the adrenal of the decapitated mouse tended to vary directly with the length of the experimental period, i.e., with the length of period of absence of head. The lipid-free zone

Received July 6, 1960.

TABLE 1. AVERAGE BODY WEIGHTS AND ADRENAL VOLUMES IN DECAPITATED FETUSES AND IN CONTROLS

Duration of experiments (days)	No. of litters	Fetuses				Body wt. (gm.) Av.†	Left adr. volume ($mm^3 \times 10^2$) Av.	$\left(\frac{\text{Adr. vol. } (mm^3 \times 10^2)}{\text{Body wt. (gm.)}}\right)$	
		Group	Number*	Day of decapitation	Day of sacrifice			Av.	C−D/C
1	3	1	3 D	16	17	0.34	5.0	14.7	.05
			6 C			0.39	6.0	15.5	
	4	2	4 D	17	18	0.51	5.6	10.9	.17
			11 C			0.45	5.9	13.2	
	4	3	5 D	18	19	0.70	8.1	11.3	.17
			9 C			0.64	8.6	13.6	
2	4	4	5 D	16	18	0.47	5.5	11.7	.19
			9 C			0.49	6.9	14.4	
	3	5	3 D	17	19	0.69	7.2	10.4	.22
			10 C			0.57	7.7	13.4	
3	2	6	3 D	16	19	0.62	6.2	10.1	.25
			7 C			0.63	8.4	13.4	

* D, decapitated fetus; C, control fetus.
† Headless weight (controls decapitated just before weighing).

in the cortex of the rat adrenal was not matched, however, by a similar zone in the mouse adrenal (zone absent in decapitated fetus and in control fetus as well).

The adrenals of decapitated fetuses were generally smaller than those of controls, both in absolute volume and in the ratio "volume/headless body weight."

The microscopical study showed that in the adrenals of controls the epithelial cells in the outer cortical zone were small and closely packed; those in the intermediate zone were large and arranged in cords; the outer and intermediate zones were rather clearly defined. In the adrenals of decapitated fetuses, any demarkation between outer and intermediate zones was difficult to define; the cortical cells in the position of the intermediate zone of controls were shrunken and packed closely together.

Changes in the pattern of lipids were most marked in the intermediate zone, and this was especially true in the decapitated fetuses of Groups 5 and 6. The lipid granules in the cortical cells of the decapitated fetuses were larger and more widely separated from each other than those in the cortical cells of the litter-mate controls.

These several changes, volumetric and histologic, tended to increase progressively with an increase in the duration of the experiment. Also, they were very conspicuous in those cases in which the period of decapitation included the interval from Day 18 to Day 19 (Groups 3, 5 and 6).

The effects of decapitation upon the adrenal were smallest in Group 1. The "adrenal volume/body weight" in the experimental fetuses was only slightly smaller than that in the controls, this point being reflected by the tabulated "C-D/C" value of .05. The histological characteristics of the cortex were scarcely changed at all.

In Group 2, the experimental period was the same length as that in Group 1, one day, but the period extended from Day 17 to Day 18. The larger "C-D/C" value of .17 suggests that during the interval between Day 17 and Day 18 the developing hypophysis stimulates the growth of the adrenal cortex.

In Group 3 the same "C-D/C" value of .17 was accompanied, however, by conspicuous microscopical changes in the cortex, especially in the lipid pattern in the intermediate zone. In the decapitated fetuses the lipid granules were larger and more widely separated from each other than were those in the controls. This supports the view that the hy-

pophyseal control of cortical secretory activity is greater between Day 18 and Day 19 than that between Day 17 and Day 18 because the cortical lipid granules are considered to be intimately associated with the secretory function of the cortex. The fact that Groups 2 and 3 had the same "C-D/C" values suggests that the adrenal may have to some extent the capacity of "self-growth" (some growth without benefit of hypophyseal hormone).

In Groups 3, 5 and 6, all sacrificed on Day 19, the lipid granules in the cortex were very large. Considering this and the other observations, it seems probable that during fetal life the action of the hypophysis upon the adrenal cortex is first mainly on the size of the adrenal and later mostly on the secretory function of the adrenal.

DISCUSSION

According to Cohen (4) who studied rat fetuses, the experimental absence of the head between Days 18 and 20 or between Days 19 and 21 produced large damaging effects upon the adrenal, but a similar absence of head between Days 16 and 19 or between Days 20 and 22 produced no more than slight effects. Cohen (4) concluded that the stimulating action of the hypophysis upon the adrenal reaches a peak between Days 19 and 21 and then declines during the remaining days of pregnancy.

In the present studies of mice, the adrenal-stimulating activity of the hypophysis seems to increase gradually up to Day 19 of pregnancy. The experiments were not designed to determine the extent of this activity during the period between Day 19 and term. It seems likely that it decreases just before term, however, because the volume of the adrenal is reduced at birth (10).

The conclusion that in the mouse fetus the adrenal is under the stimulating influence of the hypophysis up to the 19th day of pregnancy is supported by two related findings. First, in the normal fetus ascorbic acid was first observed on Day 19, a time when an hypertrophy of the mitochondria in the cortical cells also was observed (11). Second, maternal adrenalectomy reduced the quantity of lipid and the size of the lipid granules in the adrenal cortex of the fetus (10).

Acknowledgment

I gratefully acknowledge the technical assistance of Mr. G. Hoshino, a member of the Department of Veterinary Anatomy, for decapitation of the mouse fetuses. Also, I am indebted to Dr. L. J. Wells, University of Minnesota, for revising the English of the text.

YASUNOBU EGUCHI

Department of Veterinary Anatomy
College of Agriculture
University of Osaka Prefecture
Sakai City, Osaka, Japan

REFERENCES

1. WELLS, L. J.: *Proc. Soc. Exp. Biol. and Med.* **68:** 487. 1948.
2. KITCHELL, R. L. AND L. J. WELLS: *Anat. Rec.* **112:** 561. 1952.
3. JOST, A., R. JACQUOT AND A. COHEN: *C. R. Soc. Biol.* **149:** 1319. 1955.
4. COHEN, A.: *Arch. Anat. microsc. et Morph. exp.* **40:** 265. 1955.
5. CHRISTIANSON, M. AND I. C. JONES: *J. Endocrinol.* **15:** 17. 1957.
6. NOUMURA, T.: *Jap. J. Zool.* **12:** 279. 1959.
7. JOST, A.: *C. R. Soc. Biol.* **142:** 273. 1948.
8. KONDO, K., K. HIMENO, H. IKOMA AND T. KATSURAGI: *Bull. Nation. Inst. Agric. Sci.* (Chiba, Japan). Ser. G. **7:** 9. 1953 (in Japanese with English summary).

9. EGUCHI, Y.: *Zool. Mag.* **69**: 250. 1960 (in Japanese with English résumé).
10. EGUCHI, Y.: *Embryologia* **5**: 206. 1960.
11. EGUCHI, Y. AND Y. HASHIMOTO: *Acta anat. Nippon.* **32**: 601. 1957. (in Japanese with English summary).

CHANGES IN OSMOLARITY IN THE CEREBRAL VENTRICLES AND DIURESIS*

ABSTRACT

In dogs under light anesthesia the cerebral ventricles were perfused with normotonic, hypotonic or hypertonic solutions. Several hours after the beginning of the experiments pronounced influences on urine flow were observed in those animals which showed a good kidney function. Perfusion of the cerebral ventricles with a hypotonic solution produced after about 20 minutes a marked increase in urine flow. Mean increases in diuresis of 351%, 555% and 378% were observed. Returning to a perfusion with an iso- or hypertonic solution diuresis again decreased.

In his classic studies VERNEY (1, 2) demonstrated that the injection of small amounts of hypertonic salt solutions in the carotid artery of trained unanesthetized dogs provokes a marked inhibition of the diuresis which occurs after a water load. From his experiments he concluded that the secretion of the antidiuretic hormone (ADH) of the neurohypophysis is under the control of so called osmoreceptors localized somewhere in the brain. Increase in the osmotic pressure of the plasma would stimulate the ADH secretion through these receptors. He assumed that, inversely, the decrease in the osmotic pressure produces an inhibition of the ADH release. In the present experiments the influence on diuresis of changes in the osmotic pressure at the level of the central nervous system has been investigated.

METHODS

The experiments were carried out in dogs under light chloralose anesthesia. After induction of anesthesia the animals received by stomach tube a water load equal to 2-3% of their body weight. A slow intravenous infusion of isotonic glucose solution (5 drops/min.) was also maintained during the experiment. The urine was continuously collected through plastic catheters introduced in both ureters and measured at regular time intervals (every 5 to 10 min.). A perfusion of the cerebral ventricles was made using a technique which has been described in earlier publications (3, 4). The solutions used for the perfusion of the cerebral ventricles had basically the composition of the normal cerebrospinal fluid. However, by alterations in their NaCl concentration, they were made isotonic, hypotonic (40% below isotonicity) or hypertonic (20% above isotonicity). The experiment itself was started 6 to 8 hours after the induction of narcosis and the preparation of the animals with the purpose of diminishing as much as possible the influence of the narcosis on the renal function. In relation with this procedure, we may mention that GAUER *et al.* (5) also obtained their results concerning the influence of negative pressure respiration on diuresis only several hours after the start of their experiments.

RESULTS

In the 9 experiments carried out so far, the following results were obtained. In four of the animals the diuresis in the control conditions was constantly very low (less than 2 cc. per 5 min. during the cerebral perfusion with the normal isotonic solution) and no increase in urine flow was observed in the hours which followed the preparation of the animals. Changing the perfusion from the normal solution to a hypotonic fluid in these animals did not produce any reaction.

In the other 5 animals in which the urine flow during the continued perfusion of the

* Preliminary communication: Arch. internat. Physiol. Bioch., 1960, **68**, 372.
Received July 18, 1960.

isotonic fluid increased progressively in the hours following the preparation of the animals, striking observations were made when the osmotic pressure in the cerebral ventricles was changed. Proceeding from the perfusion with the normal isotonic solution to that with the hypotonic fluid, a marked increase in urine flow was observed after about 20 minutes of perfusion. Returning to the perfusion with the normal isotonic fluid diuresis again decreased progressively. When instead of the isotonic fluid, the hypertonic solution was perfused after the hypotonic solution, the decrease of the diuresis was very rapid and drastic (Fig. 1). This phenomenon could be regularly reproduced by returning to the perfusion with the hypotonic solution. Usually the first diuretic responses on perfusion with the hypotonic solution were not very marked. But the reac-

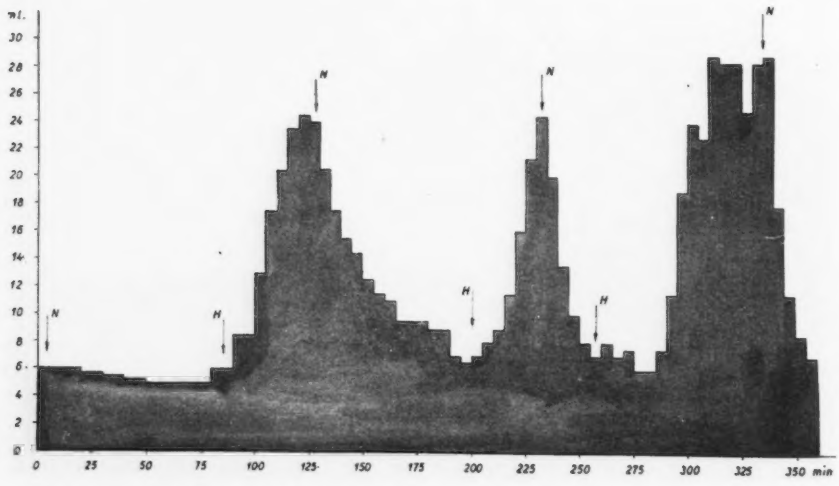

FIG. 1. Dog 21 kg. Urine flow (cc. per 5 min.) on successive perfusions of the cerebral ventricles with hypertonic (N) and hypotonic (H) solutions. Start of the experiment 8 hours before 0 time.

tion became more pronounced on successive perfusion periods with the hypotonic fluid so that mean increases in diuresis of 351%, 555% and 378% were observed. The results of the last 3 perfusions with the hypotonic solution are summarized in Table 1. It was also observed that on continued perfusion with the hypotonic solution the diuresis usually was not kept at its maximal level but that it decreased again spontaneously after about 15 minutes. The urine which was collected during the diuresis was very diluted, indicating a water diuresis. In some cases the total amounts of Na, K, Cl excreted during the water diuresis were slightly lower than during the control periods, sometimes however this did not occur.

During the perfusion of the hypotonic solutions, no other important changes in the animals were observed: the heart rate did not change markedly, the blood pressure showed a slight tendency to decrease somewhat.

These observations confirm that in the central nervous system structures sensitive to the osmolarity of NaCl concentration influence the neurohumoral control of the water excretion by the kidney. The time lag of 20 to 30 minutes between the start of perfusion with the hypotonic solution and the diuretic response probably represents the time

TABLE 1. URINE FLOW (CC. PER 5 MIN.) AND Na AND K CONCENTRATIONS IN THE URINE ON SUCCESSIVE PERFUSIONS OF THE CEREBRAL VENTRICLES WITH NORMO- OR HYPERTONIC (CONTROL) AND HYPOTONIC SOLUTIONS. FIRST DIURETIC RESPONSES OF THE ANIMALS 8 TO 10 HOURS AFTER THE BEGINNING OF THE EXPERIMENTS

Dog: weight kg.	Control perfusion			Hypotonic perfusion			% increase in urine flow	Control perfusion			Hypotonic perfusion			% increase in urine flow	Control perfusion			Hypotonic perfusion			% increase in urine flow
	Urine flow cc./5 min.	Na m.eq./l.	K m.eq./l.	Urine flow cc./5 min.	Na m.eq./l.	K m.eq./l.		Urine flow cc./5 min.	Na m.eq./l.	K m.eq./l.	Urine flow cc./5 min.	Na m.eq./l.	K m.eq./l.		Urine flow cc./5 min.	Na m.eq./l.	K m.eq./l.	Urine flow cc./5 min.	Na m.eq./l.	K m.eq./l.	
24	7	180	—	36	24	—	414	7	188	—	39	28	—	457	8	180	—	27	—	—	237
21	2	340	98	15	20	5	650	2	235	137	28	—	—	1300	2	267	80	27	—	—	1250
21	6	37.5	89	24	10	17.5	300	6	107	100	24	25	18	300	6	166	49	29	8.5	5	383
19	4	248	69	17	74	13	325	2	177	86	40	7.5	1.5	1900	2.5	66	64	28	4.5	3.5	1020
13	6.5	222	28	23	38	3.5	254	6.5	268	30	23	84	7	254	8.5	232	30	18	95	10	112
Mean	5.1	205.5	71.0	23	33.2	9.7	351	4.7	195	88.2	30.8	36.1	8.8	555	5.4	182.2	55.7	25.8	36.0	6.2	378

necessary for inactivation of the ADH present prior to the onset of perfusion with the hypotonic solution (2).

Measurements indicated that the fluid which perfused the ventricles was practically completely recovered, so that no extra uptake in the circulation of fluid during the perfusion with the hypotonic solution has to be taken into consideration as a factor responsible for the observed diuretic response. These experiments show the possibility of a new approach to the study of the role of the central nervous system in the control of the metabolism of water and electrolytes.

I. LEUSEN, E. LACROIX

Laboratory of Physiopathology,
University of Ghent, Belgium

REFERENCES

1. VERNEY, E. B.: *Proc. Royal Soc. London* (B) **135:** 25. 1947.
2. VERNEY, E. B.: *Lancet* **11:** 1237, 1295. 1957.
3. LEUSEN, I.: *J. Physiol.* (London) **110:** 319. 1950.
4. LEUSEN, I.: *Am. J. Physiol.* **176:** 39. 1954.
5. GAUER, O. H., J. P. HENRY, H. O. SIEKER AND W. E. WENDT: *J. Clin. Invest.* **33:** 287. 1954.

A REVALUATION OF THE EFFECT OF PARATHYROID HORMONE ON MUCOPOLYSACCHARIDE METABOLISM

ABSTRACT

A calculation, with its theoretical basis, is provided which suggests that the elevation in serum mucoproteins observed after PTE could not be attributable to resorption of bone matrix.

Several recent studies have exhibited a rise in serum mucoprotein and sulfate fractions after administration of Eli Lilly prepared Parathyroid Extract (PTE) to rats (1–4). It has been suggested that these elevations were a manifestation of mucopolysaccharide breakdown of bone matrix subsequent to enhanced bone resorption. By treating PTE with formaldehyde, Shetlar *et al.* (5) cited preliminary evidence for a dissociation of its serum mucoprotein elevating effect and calcium mobilizing activity. Perforce, the conclusion was entertained that the former effect may be attributed to extraneous protein in the PTE rather than to the hormone itself.

Were one to assume that demineralization of bone must precede any attack on the matrix, a view shared by the Neumans (6) in their summation of available evidence, it may be shown that the rise in serum mucoprotein observed after PTE could not have originated from bone matrix. One can calculate, within reasonable limits, that the increase in serum calcium which would have to accompany that observed for serum mucopolysaccharides would be fantastically high.

A sample calculation is presented for estimation of the serum calcium rise which would have to accompany the rise of serum mucoprotein-carbohydrate observed by Engel (1) twenty-four hours after administration of 300 units of PTE to rats.

a) The elevation of serum mucoprotein-carbohydrate above normal levels was about 16 mg.%.

b) The average weight of the animals used was 200 gm. Assuming that the extracellular fluid and plasma compartments are 20% of body weight, 6.4 mg. of carbohydrate would have to be removed from bone matrix to account for the observed rise in serum mucoprotein-carbohydrate.

c) According to Eastoe *et al.* (7), mucopolysaccharide-protein complex contributes 0.24% to the total weight of bone. The loss of mucopolysaccharide-carbohydrate given in (b) would necessitate the loss of at least 2666 mg. of bone (organic plus inorganic constituents).

d) Since calcium contributes a minimum of 25% of the total weight of bone, the resorption of 2666 mg. would contribute an extra 660 mg. of calcium to be distributed throughout the extracellular fluid compartments and/or to be excreted.

e) The increased excretion of calcium by the kidneys during the twenty-four-hour period after PTE administration to rats observed by Talmage *et al.* (8) was negligible as compared to the estimated amount in (d) released from bone.

f) The increase of serum calcium would have to be on the order of 3000 mg.% to account for the elevation of serum mucoprotein-carbohydrate.

Shetlar *et al.* (2) observed a serum mucoprotein-carbohydrate rise of about 10 mg.% twenty four hours after PTE administration, which would necessitate a serum calcium increase close to 1800 mg.%. Not enough published data are available from the studies of Bradford *et al.* (3) and Bronner (4), in which elevated serum sulfate fractions were observed, to permit similar calculations to be made.

The hypothesis is advanced that extraneous material in PTE influences normal production and/or destruction of serum mucoproteins by the liver. A reasonable basis for this suggestion is provided by experiments utilizing isolated perfused rat livers and

Received October 1, 1960.

eviscerated animals (9) which indicate that 90 to 95% of the serum mucoproteins are synthesized by the liver. The problem of the relationship between parathyroid activity and serum mucoprotein levels may well be resolved by studies utilizing highly purified parathyroid hormone (10).

ERNEST HAUSMANN

Department of Pharmacology
School of Dentistry and School of Medicine
University of Buffalo
Buffalo, New York

REFERENCES

1. ENGEL, M. B.: *Arch. Path.* **53**: 339. 1952.
2. SHETLAR, M. R., P. R. HOWARD, W. JOEL, D. C. BOWLING AND C. L. SHETLAR: *Am. J. Physiol.* **195**: 535. 1958.
3. BRADFORD, R. H., R. P. HOWARD, W. JOEL AND M. R. SHETLAR: *Biochem. and Biophys. Res. Comm.* **1**: 350. 1959.
4. BRONNER, F.: *Am. J. Physiol.* **198**: 605. 1960.
5. SHETLAR, M. R., H. PALMER AND E. C. REIFEINSTEIN: *Fed. Proc.* **13**: 296. 1954.
6. NEUMAN, W. F. AND M. NEUMAN: The Chemical Dynamics of Bone Mineral, The University of Chicago Press, Chicago, Ill. 1958.
7. EASTOE, J. E. AND B. EASTOE: *Biochem. J.* **57**: 453. 1954.
8. TALMAGE, R. V. AND F. W. KRAINTZ: *Proc. Soc. Expt. Biol. and Med.* **87**: 263, 1954.
9. MILLER, L. L.: Resumes Communs. 3rd Congr. intern. biochem., Brussels, 45. 1955.
10. AURBACH, G. D.: *J. Biol. Chem.* **234**: 3179. 1959.

THE HYPOGLYCEMIC ACTION OF SIMIAN AND BOVINE GROWTH HORMONE IN HYPOPHYSECTOMIZED RHESUS MONKEYS[1]

ABSTRACT

Simian growth hormone when administered to fasting hypophysectomized rhesus monkeys causes a marked hypoglycemia leading to convulsions and coma. The injection of bovine growth hormone to such animals is without effect. Simian growth hormone does not influence the blood glucose concentrations of fed hypophysectomized or fasted normal monkeys.

These findings suggest that the insulin-like action of growth hormone is an inherent property of the growth hormone molecule, since it shares the "species specificity" exhibited by other criteria of growth hormone activity.

Repeated reports of a hyperglycemic action of non-primate growth hormone preparations in man (1, 2, 3, 4) have suggested the possibility that these preparations, while generally ineffective in promoting nitrogen retention in primates, may nevertheless produce alterations in carbohydrate metabolism characteristic of those associated with the action of growth hormone in lower mammals.

Although sheep prolactin preparations have, on occasion, produced a reduction in glucose tolerance and insulin sensitivity in hypophysectomized monkeys, purified bovine and porcine growth hormone preparations were invariably without effect in this regard (5). The administration of bovine growth hormone to hypophysectomized and normal monkeys, however, has led in several instances to a decrease in blood sugar concentration and increases in insulin sensitivity and in glucose tolerance (5). The possibility that

Received November 15, 1960.

[1] Aided by grants A-3754 and A-292 from the National Institutes of Health, United States Public Health Service.

bovine growth hormone may share with simian growth hormone the capability of inducing a hypoglycemia in fasting hypophysectomized monkeys was investigated by means of the following experiments.

Materials and Methods

Twenty-one experiments were performed in 2 normal and 4 hypophysectomized male rhesus monkeys. The latter were utilized from 1 to 4 years following the operation, the completeness of which was evidenced by cessation of growth, failure of sexual development, increased insulin sensitivity and glucose tolerance, as well as diminished thyroid function. The care and feeding of these hypophysectomized monkeys have been described in detail (5).

The normal animals were fasted for 18 hours before hormone injection, while food was withheld from the hypophysectomized monkeys 3 hours before initiation of experiments. Simian[2] (0.5–1.0 mg./kg.) and bovine[3] growth hormone (1.0–2.0 mg./kg.) were given as a single intramuscular injection at 0 time of the experiment. Blood samples were obtained at 30-minute intervals thereafter for 3 hours.

The non-fasted animals were allowed access to food up to the time of hormone injection.

Control experiments were performed in an identical manner, saline being substituted for the growth hormone injections.

Blood glucose concentration was determined by the Somogyi modification (6) of the Nelson (7) method.

Results and Discussion

Simian growth hormone (0.5–1.0 mg./kg.) when given as a single intramuscular injection to fasting hypophysectomized monkeys, produced a marked hypoglycemia resulting in convulsions and coma approximately 2 hours after the injection in 3 of the 4 hypophysectomized animals studied. Bovine growth hormone given at twice the dose

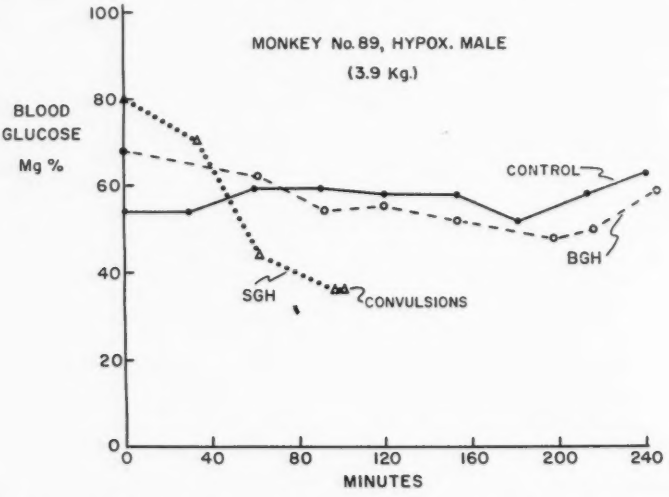

Fig. 1. Effects of a single intramuscular injection (given at 0 time) of simian growth hormone (SGH) and bovine growth hormone (BGH) on the blood glucose concentration of a fasting hypophysectomized monkey. SGH = 0.5 mg./kg.; BGH = 1 mg./kg.; Control = Saline injection.

[2] The simian growth hormone (lots M425B and M334B-41B) was prepared and kindly provided by Dr. A. E. Wilhelmi.

[3] The bovine growth hormone NIH–BGH–1, was a gift of the Endocrinology Study Section, National Institutes of Health.

used for the simian preparation, and under identical conditions, had no hypoglycemic effect. The insulin-like action of the simian hormone was abolished when the hypophysectomized animals were permitted access to food up to the time of hormone injection. A representative series of experiments in a single hypophysectomized animal is illustrated in Figure 1.

Neither simian nor bovine growth hormone had a significant hypoglycemic effect when administered to normal, fasting monkeys.

These findings suggest that the well documented insulin-like action of growth hormone (see 8 for review) is an inherent property of the growth hormone molecule since it shares the "species specificity" exhibited by all the other criteria of growth hormone activity studied to date (5).

E. KNOBIL, G. R. BEST AND H. M. GOODMAN[4]

Department of Physiology
Harvard Medical School
Boston, Massachusetts

[4] Predoctoral Research Fellow of the National Cancer Institute, United States Public Health Service.

REFERENCES

1. SHORR, E., A. C. CARTER, B. J. KENNEDY AND R. W. SMITH: *Tr. A. Am. Physicians* **66:** 114. 1953.
2. KINSELL, L. W., S. MARGEN, J. W. PARTRIDGE, G. D. MICHAELS, H. E. BALCH AND J. P. JAHN: *J. Clin. Endocrinol. and Metab.* **14:** 110. 1954.
3. FROESCH, E. R., W. F. GANONG, H. A. SELENKOW, N. GOODALE, A. E. RENOLD AND G. W. THORN: *Diabetes* **6:** 515. 1957.
4. BECK, J. C., E. E. MCGARRY, I. DYRENFURTH, R. O. MORGEN, E. D. BIRD AND E. H. VENNING: *Metabolism* **9:** 699. 1960.
5. KNOBIL, E. AND R. O. GREEP: *Rec. Prog. Hormone Res.* **15:** 1. 1959.
6. SOMOGYI, M.: *J. Biol. Chem.* **160:** 60. 1945.
7. NELSON, N.: *J. Biol. Chem.* **153:** 375. 1944.
8. DE BODO, R. C. AND N. ALTSZULER: *Physiol. Rev.* **38:** 389. 1958.

THE ENDOCRINE SOCIETY

The Forty-Third Annual Meeting of the Endocrine Society will be held in the Hotel Biltmore, New York, N. Y., Thursday, Friday and Saturday, *June 22, 23 and 24, 1961.*

All members are urged to make their hotel reservations immediately. The Biltmore will hold 300 bedrooms for members until May 1, 1961, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 26 for the American Medical Association and other meetings, it is imperative that your reservations be made from June 26 on directly through the A.M.A. Housing Bureau.

Final program, membership card and advance registration forms will be sent on May 1, 1961 to members whose current dues have been paid.

1961 AWARDS

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958, Dr. Wilbur W. Swingle in 1959 and Dr. Emil Witschi in 1960. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year

the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, 1959 Dr. Gordon L. Farrell and 1960 Dr. Don H. Nelson. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

THIRTEENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

UNDER THE CO-SPONSORSHIP OF THE ENDOCRINE SOCIETY AND THE NATIONAL INSTITUTES OF HEALTH
BETHESDA, MARYLAND
OCT. 2–6, 1961

A comprehensive review of clinical endocrine problems and current research activity in these areas will be presented.

For further information write to:

Dr. Roy Hertz
National Institutes of Health
Building 10
Bethesda 14, Maryland.

The fee will be $100.00 for physicians with a reduction to $30.00 for Residents and Fellows. Enrollment limited to 100.

TRAINING PROGRAM IN THE PHYSIOLOGY OF REPRODUCTION

In recognition of the world-wide need for scientific training in reproductive physiology, the Worcester Foundation for Experimental Biology plans to continue the postdoctoral program in this field under a grant from the Population Council. Starting in 1962, the annual program will commence on February first.

Fellowships for the program will be awarded to candidates possessing the Ph.D. or M.D. degrees, or their equivalents. These Fellowships will carry a stipend of $5,500 per annum and will be for a twelve-month period. An allotment will also be made for round-trip travel to Shrewsbury Massachusetts. Application forms may be secured from the Program Director of the course in the Physiology of Reproduction, and should be returned not later than June first. They will be processed by an advisory and selection committee comprised of experts, and the Fellowship awards will be announced by September 15.

Individuals interested in participating in the program may communicate with Doctor Celso-Ramon Garcia, Program Director, at any time. However, applications will not be acted upon prior to the given date for the particular year selected. Whenever possible, arrangements should be made for an interview with a member of the selection committee. The members of the advisory and selection committee are:

Doctor Gregory Pincus
Worcester Foundation for Experimental Biology
Shrewsbury, Massachusetts

Doctor Warren O. Nelson
The Population Council, Inc.
Rockefeller Institute
66th Street & York Avenue
New York 21, New York

Doctor V. R. Khanolkar
Indian Cancer Research Centre
Parel, Bombay 12, India

Doctor Roberto Caldeyro-Barcia
Servicio de Fisiologia Obstetrica
Facultad de Medicine
Avenida Gral. Flores, 2125
Montevideo, Uruguay

Doctor Takeshi Nakao
Tokyo Jikei-kai School of Medicine
Department of Pharmacology
Atago-cho, Shiba, Minato-ku
Tokyo, Japan

Doctor M. C. Shelesnyak
Weizmann Institute of Science
P. O. Box 26
Rehovoth, Israel

The program will consist of a course of lectures and laboratory work. During the latter part of the year, each Fellow will have the opportunity to participate in a research project pertinent to his interests and within the capacity of the program.

The lecture series will be given by the Program Director, various members of the staff of the Worcester Foundation, and guest experts. The lectures and accompanying laboratory work will cover various phases of reproductive processes, particularly in mammals. Subjects to be treated will involve a review of reproductive processes in the male, including consideration of spermatogenesis, hormonogenesis, the control of testis function, and the functional endocrinology of testis hormones. Extensive attention will be given to oogenesis, ovulation, and fertilization in the female, factors affecting the entire course of pregnancy and embryonic development, as well as the postnatal physiology of the female. Additional topics will include the role of extra-reproductive tract organs in reproductive processes, genetic and nutritive factors in reproduction, and the control of fertility and sterility.

Laboratory exercises will include the study of characteristic reproductive phenomena in various species, analytic procedures employed in determining the chemistry of reproductive organs, the biochemistry of spermatozoa and ova, and hormone analyses pertinent to gonad function, to sex cycles, to gestation and lactation.

GORDON RESEARCH CONFERENCE

CHEMISTRY, PHYSIOLOGY AND STRUCTURE OF BONES AND TEETH

10–14 July 1961
Kimball Union Academy
Meriden, New Hampshire

William P. Norris, Chairman
Roy V. Talmage, Vice Chairman

10 July

Selected Communications
Robert E. Rowland, Chairman
Evolution of Bone as a Tissue
Marshall R. Urist, Chairman
Robert H. Denison. The Habitat of the Earliest Vertebrates.
Eric Stensio. The Skeleton of the Early Vertebrates

11 July

Evolution of Bone as a Tissue (Continued)
Marshall R. Urist, Chairman
Alfred S. Romer. Vertebrate Paleontology
T. Dale Stewart. The Last Phase of Human Evolution
Vasculature and Hemodynamics of Bone
Robert B. Duthie, Chairman
J. Trueta. Anatomical Considerations of Circulation

12 July

Vasculature and Hemodynamics of Bone (Continued)
Robert B. Duthie, Chairman
Donald A. McDonald. Hemodynamics of Blood Flow
Friedrich Wassermann. The Problem of Transport Between Blood and Bone
James B. Wray. The Response of Periosteal Vessels to Certain Vasomotor Agents

13 July

Physical and Chemical Reactions of Bone Mineral
William F. Neuman, Chairman
Eubert J. Daniel. Histochemical and Electron-Optical Analyses of Calcifying Systems: Part 1. Embryogenesis, Part 2. Maturation.
Howard M. Myers. Surface Properties of Apatite
Aaron S. Posner. Calcium-Hydrogen Balance in Synthetic Biological Apatites
Defects in Mineralization and in Mineral Reserve
Robert P. Heaney, Chairman
Robert Steendijk. Observations on Calcification Affected by Intravenous Administration of Inorganic Phosphate
Leo Lutwak. Osteoporosis and Its Treatment, as Evaluated by Calcium-45

14 July

Q Defects in Mineralization and in Mineral Reserve (Continued)
ROBERT P. HEANEY, Chairman
JENIFER JOWSEY. The Structure of Bone in Relation to Age and Osteoporosis
EUGENE EISENBERG. Bone Formation and Metabolism in Relation to Osteoporosis and the Aging Process

Attendance. Requests for attendance at the conferences, or for additional information, should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From 12 June to 1 September 1961 mail for the office of the Director should be addressed to Colby Junior College, New London, New Hampshire.

Registration and Reservations. Attendance at the Conferences is by application. Individuals interested in attending the conferences are requested to send their applications to the Director at least two months prior to the date of the Conference.